Cyclic di-GMP integrates functionally divergent transcription factors into a regulation pathway for antioxidant defense

Weihui Li, Lihua Hu, Zhiwei Xie, Hui Xu, Meng Li, Tao Cui and Zheng-Guo He[*]

National Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

ABSTRACT

Cyclic diguanylate monophosphate (c-di-GMP) is a global signaling molecule that modulates diverse cellular processes through its downstream receptors. However, no study has fully clarified the mechanisms by which c-di-GMP organizes functionally divergent regulators to drive the gene expression for coping with environmental stress. Here, we reported that c-di-GMP can integrate two functionally opposite receptor transcription factors, namely, LtmA and HpoR, into a pathway to regulate the antioxidant processes in *Mycobacterium smegmatis*. In contrast to HpoR, LtmA is an activator that positively regulates the expression of redox gene clusters and the mycobacterial H_2O_2 resistance. LtmA can physically interact with HpoR. A high level of c-di-GMP stimulates the positive regulation of LtmA and boosts the physical interaction between the two regulators, further enhancing the DNA-binding ability of LtmA and reducing the inhibitory activity of HpoR. Therefore, upon exposure to oxidative stress, c-di-GMP can orchestrate functionally divergent transcription factors to trigger antioxidant defense in mycobacteria. This finding presents a noteworthy example of how a bacterium remodels its transcriptional network via c-di-GMP in response to environmental stress.

INTRODUCTION

Cyclic diguanylate monophosphate (c-di-GMP) is an important and well-conserved second messenger in bacteria (1,2). Over the past decades, studies have confirmed that c-di-GMP can act as a global signaling molecule that can regulate bacterial physiological processes, such as biofilm formation (3,4), motility (5,6), cell cycle progression (7,8), and virulence (9–12). The physiological function of c-di-GMP has been recently associated with the bacterial an-

tioxidant regulation in mycobacteria (13). Essentially, c-di-GMP drives bacteria to perform physiological adaptations through its downstream receptors (1,14–16), including PilZ proteins, GGDEF and/or EAL domain-containing proteins, and riboswitches (7,17–21). Transcription factors are direct c-di-GMP effectors that extend the regulatory functions of c-di-GMP to various physiological processes (9,10,22–24). However, no study has elucidated the mechanism by which c-di-GMP, as a global signaling molecule, organizes these different receptors to adaptively drive gene regulation.

Oxidative stress is unavoidable for nearly all organisms and is a common extracellular and intracellular environment cue for bacteria. To rapidly adapt to stress, bacteria must timely deliver signals to the enhanced expression of antioxidant enzymes. *Mycobacterium smegmatis* is a slow-growing bacterium with a unique antioxidant capacity. However, no oxidative stress regulators, such as SoxR and OxyR homologs, have been reported for this bacterium to date. Rather, the bacterium contains the c-di-GMP signaling system and c-di-GMP metabolic enzymes (25,26). Two of the only known c-di-GMP receptor regulators in mycobacteria were detected in this bacterium (13,27). The first characterized transcription factor was LtmA, which could directly sense the c-di-GMP signal in mycobacteria (27). LtmA acts as a positive regulator that enhances bacterial growth under antibiotic-stressful conditions (27). The other receptor transcription factor was HpoR, which was identified in *M. smegmatis* and which associated the c-di-GMP signal to bacterial antioxidant regulation (13). LtmA and HpoR are quite similar transcription factors, and the two regulators display a 43% amino acid sequence identity (Supplementary Figure S1). However, in contrast to LtmA, HpoR functions as a negative regulator. HpoR inhibits the expression of the *hpoR* operon and enhances the H_2O_2 sensitivity of mycobacteria (13). High concentrations of c-di-GMP inhibit the DNA-binding activity of HpoR and de-repress the intracellular association of HpoR with the regulatory region of the *hpoR* operon in *M. smegmatis* (13). Therefore, LtmA and HpoR present divergent regulatory

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[*]To whom correspondence should be addressed. Tel: +86 27 87284300; Fax: +86 27 8728067; Email: he.zhengguo@hotmail.com

effects on both gene expression and environmental adaptation in *M. smegmatis*. However, no study has demonstrated whether c-di-GMP can orchestrate these functionally opposite receptor transcription factors, and if so, how, to cooperatively trigger antioxidant regulation.

In this study, we reported that c-di-GMP can orchestrate LtmA and HpoR to trigger defense against oxidative stress in *M. smegmatis*. We found that both LtmA and HpoR play roles in the same regulatory pathway to control the expression of the *hpoR* operon. C-di-GMP modulated the interactions between two regulators, further enhancing the DNA-binding ability of LtmA and reducing the inhibitory activity of HpoR. This finding revealed that c-di-GMP integrates two functionally opposite regulators into a pathway to drive antioxidant regulation.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

The genes in this study were amplified through polymerase chain reaction (PCR) by using their specific primer pairs (5′-AGATGAATTCGAGTGTCCGAT CCGGCCA TG-3′ and 5′-ATATTCTAG ATCA GCTGAGCACCAGGCGCG-3′ for *ltmA*; 5′-ATAT GAATTCGCGTGTCCAGCGATG CAGTG-3′ and 5′-AGATTCTAGATCAT CGGT CCTCTCCCAGGA-3′ for *hpoR*) and templates from the genomic DNA of *M. smegmatis* mc^2 155. Several mutant genes were obtained through site-directed mutagenesis by overlapping extension PCR. Five pairs of mutagenic PCR primers were designed (5′-AGGAGACCTTCCGCA AGGTGTACCTGGACCTGGTGCG CCA-3′ and 5′-TGGCGCACCAGGTCCAGGTACACCTTGCG GAAGGTCTCCT-3′ to amplify *hpoR*mut; 5′-AACTG ATCAGTCGCGAGGGGTTCTGGCGCAGGCTGT CCGC-3′ and 5′-GCGGACAGCCTGCGCCAGAAC CCCTCGCGACTGATCAG TT-3′ to amplify *ltmA*mut; 5′-GCGTTCGCGGGCGCGGTGGATCCCGACG TCGACGCG-3′ and 5′-CGCGTCGACGTCGGGATCC ACCGCGCCCGCGAACG C-3′ to amplify *ltmA*△114–120; 5′-TGACCACGGCCCTGCTGGGCGCGGATC CCGA CGTCG ACGCGCT-3′ and 5′-AGCGCGT CGACGTCGGGATCCGCGC CCAGCAGGGC CGTGGTCA-3′ to amplify *ltmA*K120A; and 5′-GTTCGCGGG CGCG GTGGC AGCAGCCGCA GCAGCAAAGGATCCCGACGTCGA-3′ and 5′-TCGA CGTCGG GATCCTTTGCTGCTGCGGCTGCTGCC ACCGCGCC CGCGAAC-3′ to amplify *ltmA*119–120A). The amplified DNA fragments were cloned into modified pET28a expression vectors and pMV261 overexpression vectors to produce recombinant plasmids (Supplementary Table S1). The expression strains of *Escherichia coli* BL21 containing the recombinant plasmids were cultured in LB medium at 37°C until the optical density at 600 nm (OD$_{600}$) was 0.6. Then, 0.3 mM isopropyl β-D-1-thiogalactopyranoside was added to the culture, and the strains were cultured for another 4 h at 37°C. The cells were harvested, and the proteins were purified on Ni^{2+} affinity columns as described previously (28). The elution was dialyzed overnight and stored at −80°C.

Transcriptomic analysis

Transcriptomic analysis was conducted as described previously (13). Strains were grown in 7H9 medium and shaken at 160 rpm and 37°C. The cells were cultured until mid-logarithmic phase (OD$_{600}$: 1.0) and harvested. Total RNA was isolated using an RNeasy mini kit (Qiagen, Germany). Strand-specific libraries were prepared using the TruSeq® Stranded Total RNA Sample Preparation kit (Illumina, USA) in accordance with the manufacturer's instructions. In brief, ribosomal RNA was removed from total RNA by using Ribo-Zero rRNA removal beads. After purification, the mRNA was fragmented into small pieces by using divalent cations at 94°C for 8 min. The cleaved RNA fragments were copied into first-strand cDNA by using reverse transcriptase and random primers, followed by second-strand cDNA synthesis using DNA Polymerase I and RNase H. Then, the cDNA fragments underwent an end repair process, added with a single 'A' base, and ligated of the adapters. The products were purified and enriched with PCR to create the final cDNA library. The purified libraries were quantified by a Qubit® 2.0 Fluorometer (Life Technologies, USA) and validated by an Agilent 2100 bioanalyzer (Agilent Technologies, USA) to determine the insert size and calculate the mole concentration. Clusters were generated by cBot with the library diluted to 10 pM and then sequenced on the Illumina HiSeq 2500 (Illumina, USA). Library construction and sequencing were performed at Shanghai Biotechnology Corporation.

The heat map was created using the MeV (Multiple Experiments Viewer) software. The FPKM (fragments per kilobase of exon per million fragments mapped) values of the target genes in each sample were imported to the MeV software and normalized to build the heat map diagram. The heat map shows the expression of genes in different samples. Red indicates high expression, whereas green denotes low expression. The color scale above the heat map displays the threshold of the color.

β-Galactosidase activity assays

β-Galactosidase activity assays were performed in accordance with previously described procedures (13) with modifications. The experiments were conducted on *M. smegmatis* by constructing operon-*lacZ* fusions based on the expression vector of pMV261 (29). Target and control promoters were cloned into the pMV261 backbone. Then, the reporter gene *lacZ* was cloned behind the promoters. The plasmids were transformed into the *ltmA* knockout strain and the wild-type *M. smegmatis* strain to obtain the recombinant reporter strains. All recombinant strains were grown in 7H9 medium at 37°C until the log phase was reached. The bacterial cells were harvested and washed twice with PBS. β-galactosidase measurements were performed as described previously (30).

Determination of mycobacterial growths

The target recombinant strains were cultured in 7H9 medium with 50 µg/ml kanamycin at 37°C until the mid-log phase was reached. Each culture was diluted (4:100) in 100 ml of fresh 7H9 medium containing 50 µg/ml kanamycin

and the indicated concentration of H_2O_2. Then, the cultures were allowed to grow further at 37°C under shaking at 160 rpm. At different time points, the serial dilutions of the samples were plated on 7H10 plates to count the colony-forming units. Aliquots were collected at the indicated times. Each group assay was performed with three biological repeats. The error bars in the figures represent the standard deviation (SD) of the data derived from three biological replicates.

Bacterial two-hybrid assay

The BacterioMatch II Two-Hybrid System (Stratagene) was used to establish the protein–protein interactions (PPIs) between HpoR and LtmA. This system detected the PPIs through transcriptional activation, and the results were analyzed in accordance with previously published procedures (31,32). The positive growths of the cotransformants were selected on the selective screening medium plate containing 5 mM 3-amino-1,2,4-triazole (3-AT) (Stratagene), 8 μg/ml streptomycin, 15 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 50 μg/ml kanamycin. Cotransformants containing pBT-LGF2 and pTRG-Gal11^P (Stratagene) were used as the positive controls for an expected growth on the screening medium. Cotransformants containing empty vector pBT and pTRG were used as the negative controls.

Surface plasmon resonance (SPR) analysis

SPR analysis was conducted using a Biacore 3000 instrument (GE Healthcare) with CM5 and SA sensor chips. The assays were performed at 25°C. For example, in the HpoR–LtmA interaction, HpoR was immobilized onto CM5 chips, and LtmA was used as the analyte and passed over the chips. LtmA was diluted in the HBS buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 50 μM EDTA, and 0.005% BIAcore surfactant P20) at concentrations of 1.25, 2.5, 5, 10, 20 and 40 μM and injected at 10 μl/min for 5 min at 25°C. An overlay plot was produced using BIAevaluation 3.1 software to depict the HpoR–LtmA interaction. To assess the effect of c-di-GMP on the DNA-binding activity of LtmA, Biotin-labeled Ms5860p was immobilized onto the SA chips. Different concentrations of c-di-GMP (1–32 μM) were incubated with a fixed concentration of LtmA (130 nM) for 30 min at 4°C. Then, the complex was passed over the chips. An overlay plot was produced using BIAevaluation 3.1 software to depict the effect of c-di-GMP on the DNA-binding activity of LtmA.

Electrophoretic mobility shift assay (EMSA)

EMSA assays were performed in accordance with a previously described procedure with several modifications (13).The upstream regulatory sequence of the *hpoR* operon, i.e. Ms5860p (371 bp), was amplified through PCR by using a pair of primers (5′-CGAACACGCCTTTCCGGCC TGCGACG AGTTT-3′ and 5′-AGCATCTCGATGCCC GCGGTGAGCACCTTGC-3′). The DNA fragments (5 ng/μl) were labeled by fluorescein isothiocyanate and used in the EMSA assays. The protein sizes of LtmA, HpoR, LtmAmut, HpoRmut, and LtmA△114–120 were 20.5, 21.5,

16.8, 16.5 and 19.7 kDa, respectively. As regards the DNA fragments, various amounts of proteins and small molecules of c-di-GMP were contained in the EMSA reactions. First, proteins and small molecules were co-incubated for 30 min. Then, DNA substrates were added to the reactions, which were incubated for another 30 min. Finally, the mixtures were directly subjected to 5% native polyacrylamide gel electrophoresis containing 0.5× Tris–borate–EDTA buffer. Electrophoresis was performed at 150 V. Images were acquired using a Typhoon Scanner (GE Healthcare).

Quantitative chromatin immunoprecipitation

Quantitative chromatin immunoprecipitation is a quantitative PCR (qPCR) quantification approach used for chromatin immunoprecipitation (ChIP). ChIP was performed as described previously (13,33). The target recombinant strains were cultured in 7H9 medium until the mid-log phase was achieved, fixed with 1% formaldehyde, and then terminated with 0.125 M glycine. Then, the sample was sonicated on ice after the cross-linked cells were harvested and resuspended in 1 mL of TBSTT (TBS, 0.2% Triton X-100, 0.05% Tween-20). The supernatant extract was collected by centrifugation. Special antibodies or preimmune serum were added to the sample extracts under rotation for 3 h at 4°C. The complexes were immunoprecipitated with 20 μl of 50% proteinA-agarose for 1 h at 4°C. The immunocomplexes were recovered by centrifugation and resuspended in 100 μl of TE (20 mM Tris–HCl, pH 7.8, 10 mM EDTA, 0.5% SDS). Then, the cross-linking was reversed for 6 h at 65°C. The DNA samples of the input and the ChIP were purified and analyzed by qPCR.

qPCR was performed as previously described (13). Each PCR (25 μl) contained the following ingredients: 10 μl of 2 × SYBR qPCR Mix kit (Aidlab, China), 200 nM Ms5860p-specific primer pairs (5′-GATCGTCAGGTCCCGCAAC TCCT CGA CATGC-3′ and 5′-GCTGGACACATGTCC AAGTTAGCCGAGCGCG-3′), and 2 μl of the immunoprecipitated and purified DNA samples from the ChIP assay. Each reaction was performed in triplicates. Ms5860p was amplified and detected using a CFX96 instrument (BIO-RAD) with the following protocol: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 45 s, melt curve, 55°C to 99°C, 0.5°C/10 s, 25°C for 5 min. The data were analyzed with Bio-Rad CFX Manager Version 2.1. Amplification specificity was assessed by melting curve analysis. The relative quantity of Ms5860p in ChIP was normalized to the input quantity of Ms5860p. The degree of change in the relative quantity of Ms5860p was calculated using the $2^{-\triangle\triangle Ct}$ method (34). For statistical analysis, two-tailed Student's t-tests were performed.

LC–MS/MS detection of the c-di-GMP binding motif of LtmA

Exactly 10 μM LtmA proteins were co-incubated with 5 mM c-di-GMP on ice for 10 min. After ultraviolet irradiation for 30 min, the cross-linked samples were directly subjected to a 12% w/v SDS-PAGE (27,35). Then, the proteins were recovered from the gel and handled in accordance with previously published procedures (36) with modifications. The samples were digested with trypsin, and the

supernatants were transferred to a new tube. The remaining gels were extracted once with 50 μl of extraction buffer at 37°C for 30 min. The peptide extracts and the supernatant of the gel spot were combined and completely dried. The samples were re-suspended with Nano-RPLC buffer A. The online Nano-RPLC was employed on the Eksigent nanoLC-Ultra™ 2D System (AB SCIEX). The samples were loaded on a C18 nanoLC trap column (100 μm × 3 cm, C18, 3 μm, 150 Å) and washed with Nano-RPLC Buffer A (0.1% FA, 2% ACN) at 2 μl/min for 10 min. An elution gradient of 5–35% acetonitrile (0.1% formic acid) in a 90 min gradient was used on an analytical ChromXP C18 column (75 μm × 15 cm, C18, 3 μm 120 Å) with a spray tip. Data were acquired using a Triple TOF 5600 System (AB SCIEX, USA) fitted with a Nanospray III source (AB SCIEX, USA) and a pulled quartz tip as the emitter (New Objectives, USA) at an ion spray voltage of 2.5 kV, a curtain gas of 30 PSI, a nebulizer gas of 5 PSI, and an interface heater temperature of 150°C. For information-dependent acquisition, survey scans were acquired in 250 ms, and up to 35 product ion scans were collected if they exceeded the threshold of 150 counts/s with a 2^+–5^+ charge state. The total cycle time was fixed to 2.5 s. For collision-induced dissociation, a rolling collision energy setting was applied to all precursor ions. The dynamic exclusion was set to one-half of the peak width (18 s). Then, the precursor was refreshed off the exclusion list. On the basis of the combined MS and MS/MS spectra, the proteins were successfully identified based on 95% or a higher confidence interval of their scores on the MASCOT V2.3 search engine (Matrix Science Ltd., London, UK) with the following search parameters: cowlacbobacillus casel mix database; trypsin as the digestion enzyme; two missed cleavage sites; fixed modifications of Carbamidomethyl (C); partial modifications of Acetyl (Protein N-term), Deamidated (NQ), Dioxidation (W), Oxidation (M) Phospho (ST) and Phospho(Y); ±30 ppm for precursor ion tolerance and ±0.3 Da for fragment ion tolerance.

RESULTS

LtmA binds with the upstream DNA sequence of the *hpoR* operon and positively regulates its expression

LtmA is the first c-di-GMP responsive regulator characterized in mycobacteria, and it was previously demonstrated to recognize a conserved 12 bp palindromic sequence motif (GGACANNTGTCC) (27). This motif sequence was very similar to that recognized by HpoR, which was the second c-di-GMP regulator in the same mycobacterium and which regulated the expression of the *hpoR* operon (13). This finding suggests that LtmA is also involved in regulating the gene cluster and in turn the mycobacterial antioxidant ability. To verify this assumption, we first performed an EMSA experiment to examine the binding of LtmA with the upstream fragment of the operon. As shown in Figure 1A, LtmA could well bind with the Ms5860p DNA substrate because the amount of shifted DNA increased stepwise as the amount of LtmA proteins increased (0.2–1.6 μM) in the reactions. By contrast, LtmA did not bind with the Ms5860p3 DNA, in which the motif sequence was mutated (13). Then, we constructed a *ltmA*-deleted mutant

M. smegmatis strain and compared the differential expression of the *hpoR* operon between the mutant and wild-type strains through transcriptomic assays. As shown in Figure 1B, when the gene expression difference of these two strains was compared by RNA sequencing, the expression of the *hpoR* operon was downregulated in the mutant strain (Figure 1B and Supplementary Table S2). This result indicates that LtmA positively regulates the expression of the *hpoR* operon in *M. smegmatis*.
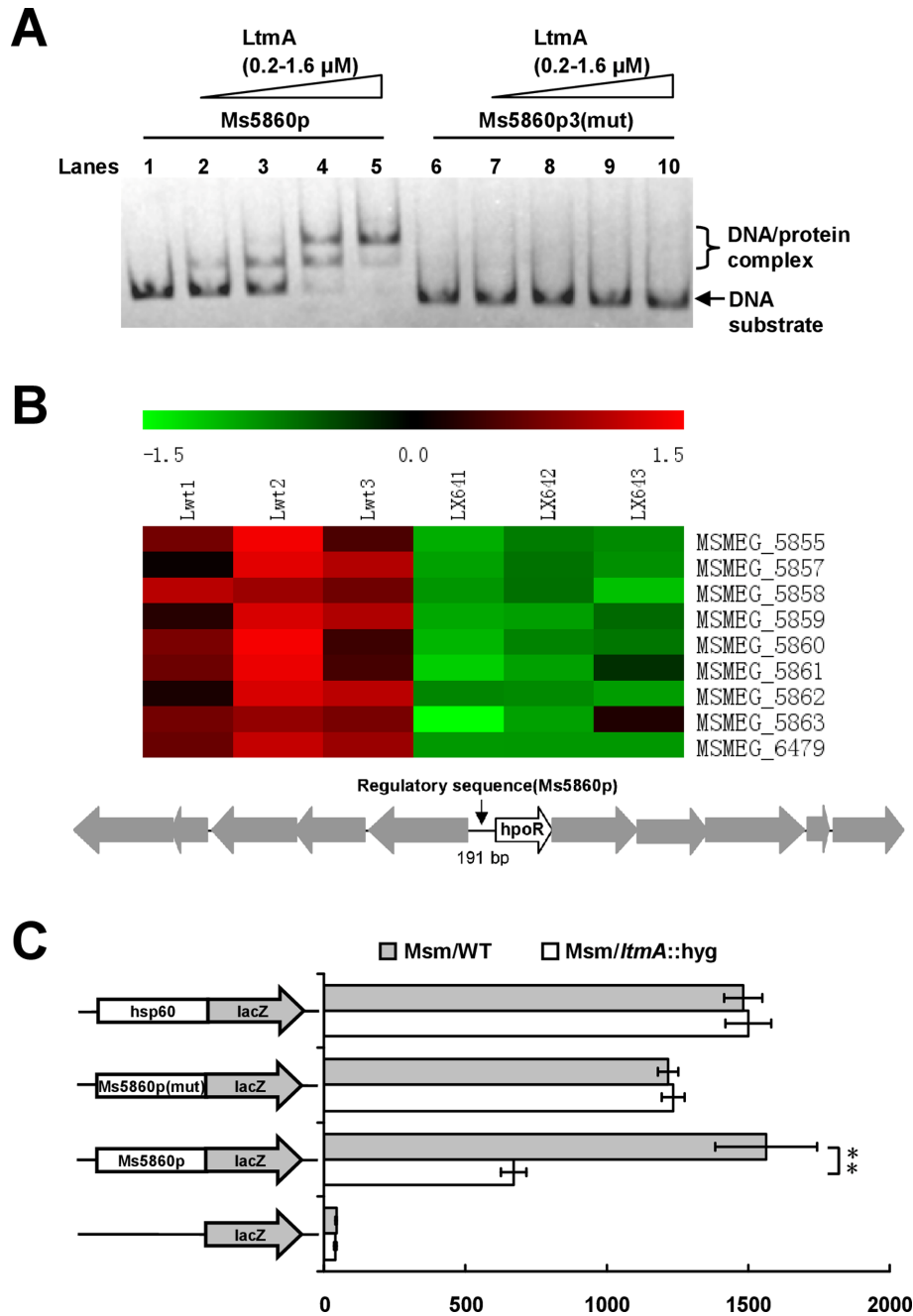
The positive regulation of LtmA on the operon was further confirmed by β-galactosidase activity assays. As shown in Figure 1C, *hsp60* strongly promoted the expression of *lacZ* in wild-type *M. smegmatis* strains relative to the non-promoter *lacZ* plasmid, indicating that the report system worked well. The expression of *lacZ* was downregulated in the *ltmA*-deleted mutant *M. smegmatis* strains when compared with the wild-type strains. When a negative control Ms5860p mutant (Ms5860p3) was used as the promoter, no significant difference was observed in the *lacZ* expression of the wild-type and mutant strains. These data indicate that LtmA positively regulates the expression of the *hpoR* operon.

Overall, LtmA can bind with the upstream DNA sequence of the *hpoR* operon and positively regulate its expression in *M. smegmatis*.

LtmA positively regulates the mycobacterial H_2O_2 resistance

The *hpoR* operon is correlated with mycobacterial H_2O_2 resistance (13), and LtmA regulates the expression of the operon, implying that LtmA would affect the antioxidant ability of *M. smegmatis*. To validate this theory, we determined the effects of different expression levels of the *ltmA* gene on the mycobacterial growth under H_2O_2 stress. As shown in Figure 2A, the bacterial counts of the wild-type strain were significantly lower than those of the *ltmA* overexpression strain at all three time points when comparing the growth of two mycobacterial strains stressed by 2.5 mM H_2O_2 for 16, 20 or 24 h, indicating that *ltmA* overexpression enhanced the mycobacterial H_2O_2 resistance. By contrast, the *ltmA*-deleted mutant strain was more sensitive to H_2O_2 than the wild-type strain (Figure 2B). No obvious growth difference was observed between the wild-type strain and the overexpression or mutant strain in the absence of H_2O_2 stress (Supplementary Figure S2A and B). These results strongly suggest that the LtmA plays a positive role in mycobacterial H_2O_2 resistance.

We further confirmed that similar to the case of HpoR, the regulation by LtmA also depended on the upstream sequence of the *hpoR* operon. As shown in Figure 2C, when *ltmA* was overexpressed in the Ms5860p promoter mutant strain (Msm Ms5860p::hyg/pMV261-*ltmA*), the bacterial counts of the recombinant strain were significantly lower than those of the Msm/pMV261-*ltmA* strains at all three time points under H_2O_2 stressful conditions, in which *ltmA* was overexpressed in the wild-type strain. No growth difference was detected between the two strains if they grew in the absence of H_2O_2 stress (Supplementary Figure S2C). These results indicate that Ms5860p is essential in the mycobacterial H_2O_2 resistance regulation by LtmA.

Figure 1. Assays for the positive regulation of LtmA on the expression of the *hpoR* operon (Ms5855-Ms5865). (**A**) EMSA assays for LtmA. Specific DNA binding activity of LtmA on the Ms5860p promoter DNA was observed when the Ms5860p DNA substrate was co-incubated with increasing amounts of LtmA (lanes 2–5). The mutant Ms5860p3 (DNA binding motifs were deleted) was used as the negative control DNA (lanes 6–10). (**B**) Heat map of the *ltmA*-regulated differential expression profile of the *hpoR* operon (Ms5855-Ms5865 gene cluster). LX641, LX642, and LX643 represent three biological replicates of differentially expressed *hpoR* operon genes in *ltmA* knockout strain, respectively. Lwt1, Lwt2, and Lwt3 represent three biological replicates of those genes in the wild-type strain, respectively. Lower panel shows the schematic of the *hpoR* operon and its regulatory region. (**C**) β-galactosidase activity assays. Data are presented as Miller units (right panel). Left column: schematic representation of each clone used to generate recombinant strains. Two asterisks (**) in the figure denote a significant difference ($P \leq 0.01$, two-tailed Student's *t*-test) between two groups. Error bars represent the standard deviation (SD) of the data derived from three biological replicates. Null promoter-*lacZ*, hsp60-*lacZ* and Ms5860p mutant (Ms5860p3) were used as controls.
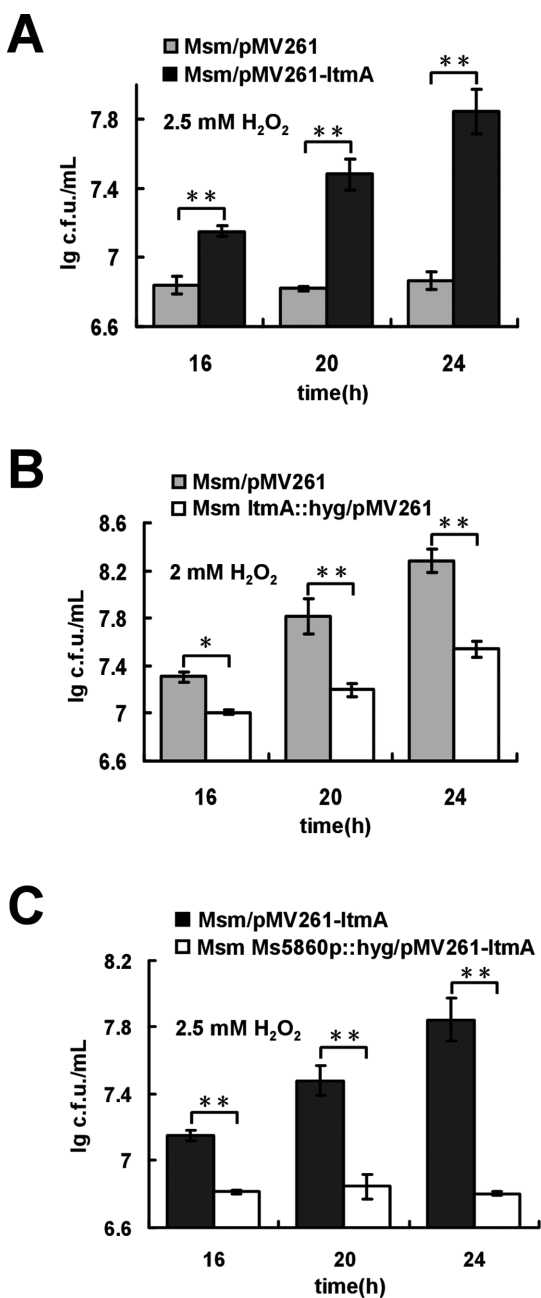
Figure 2. Assays for the effect of H_2O_2 on the growth of *M. smegmatis*. (**A**) Strains Msm/pMV261 and Msm/pMV261-*ltmA* were grown in 7H9 medium supplemented with 2.5 mM H_2O_2. (**B**) Strains Msm/pMV261 and Msm *ltmA*::hyg/pMV261 were grown in 7H9 medium supplemented with 2 mM H_2O_2. (**C**) Both Msm/pMV261-*ltmA* and Msm Ms5860p::hyg/pMV261-*ltmA* were grown in 7H9 medium supplemented with 2.5 mM H_2O_2, and the bacterial counts were determined. All error bars in the figure represent the SD of the data derived from three biological replicates. The *P*-values of the relative expression data were calculated by unpaired two-tailed Student's *t*-test using GraphPad Prism 5. Two asterisks (**) in the figure denote a significant difference ($P \leq 0.01$) between two groups.

Therefore, LtmA positively regulates the mycobacterial H_2O_2 resistance, and the regulation significantly depends on the upstream sequence of the *hpoR* operon.

c-di-GMP stimulates the DNA-binding activity of LtmA both *in vitro* and *in vivo* in *M. smegmatis*

LtmA is the first c-di-GMP responsive regulator characterized in mycobacteria, suggesting that c-di-GMP regulates the interaction between LtmA and the upstream DNA of the *hpoR* operon. We first confirmed this hypothesis by using *in vitro* EMSA assays. As shown in Figure 3A, when increasing amounts of c-di-GMP (9–900 μM) were added to the reactions (lanes 3–10), the amounts of shifted DNA substrates increased correspondingly, indicating that c-di-GMP stimulated the DNA-binding activity of LtmA (Figure 3A, lanes 3–10). By contrast, either GTP or c-di-AMP did not affect the activity of LtmA (Supplementary Figure S3). This result is consistent with our previous observation (27). To further assess whether the physiological concentration of c-di-GMP (4–8 μM) (13,27) regulates the DNA-binding activity, we conducted a SPR assay as previously described (13) to examine the dynamic interaction between LtmA and the DNA at low concentrations. When an increasing amount of c-di-GMP was added together with 130 nM LtmA and the mixture was passed over the DNA substrate-immobilized SA chip, the DNA-binding activity for LtmA continuously increased in the c-di-GMP concentration range of 1–32 μM (Figure 3B). Under a similar experimental condition, an increase in the DNA-binding activity of HpoR was previously observed in the c-di-GMP concentration range of 1–4 μM, whereas the DNA-binding activity deceased as the added amount of c-di-GMP was increased from 8 to 32 μM (13). Therefore, the regulation of c-di-GMP on the DNA-binding activity of LtmA obviously differs from that of HpoR.

We further evaluated the effect of the level of c-di-GMP on the DNA-binding activity of LtmA in the cells of *M. smegmatis*. To suppress the interference of HpoR, we expressed *ydeH*, which is an *E. coli*-derived diguanylate cyclase gene, in the *hpoR*-deleted *M. smegmatis* strain to construct a high-level c-di-GMP strain, and expressed *ydeH*(E208,209A) to construct a low-level c-di-GMP control strain. ChIP and qPCR assays revealed that LtmA could precipitate approximately ninefold higher DNA fragments of the *hpoR* operon in the high-level c-di-GMP strain than in the low-level control strain (Figure 3C), indicating that c-di-GMP can stimulate the intracellular DNA-binding activity of LtmA in *M. smegmatis*. These results were consistent with the data from our *in vitro* experiments (Figure 3A and B).

Therefore, c-di-GMP can modulate the DNA-binding activity of LtmA both *in vitro* and *in vivo* in *M. smegmatis*.

HpoR physically interacts with LtmA

The two c-di-GMP receptor transcription factors recognize a similar DNA sequence motif, implying the presence of c-di-GMP-dependent interplays between LtmA and HpoR as well as their target DNA. We detected a physical interaction between these two proteins, thereby confirming the aforementioned hypothesis. As shown in Figure 4A, the results
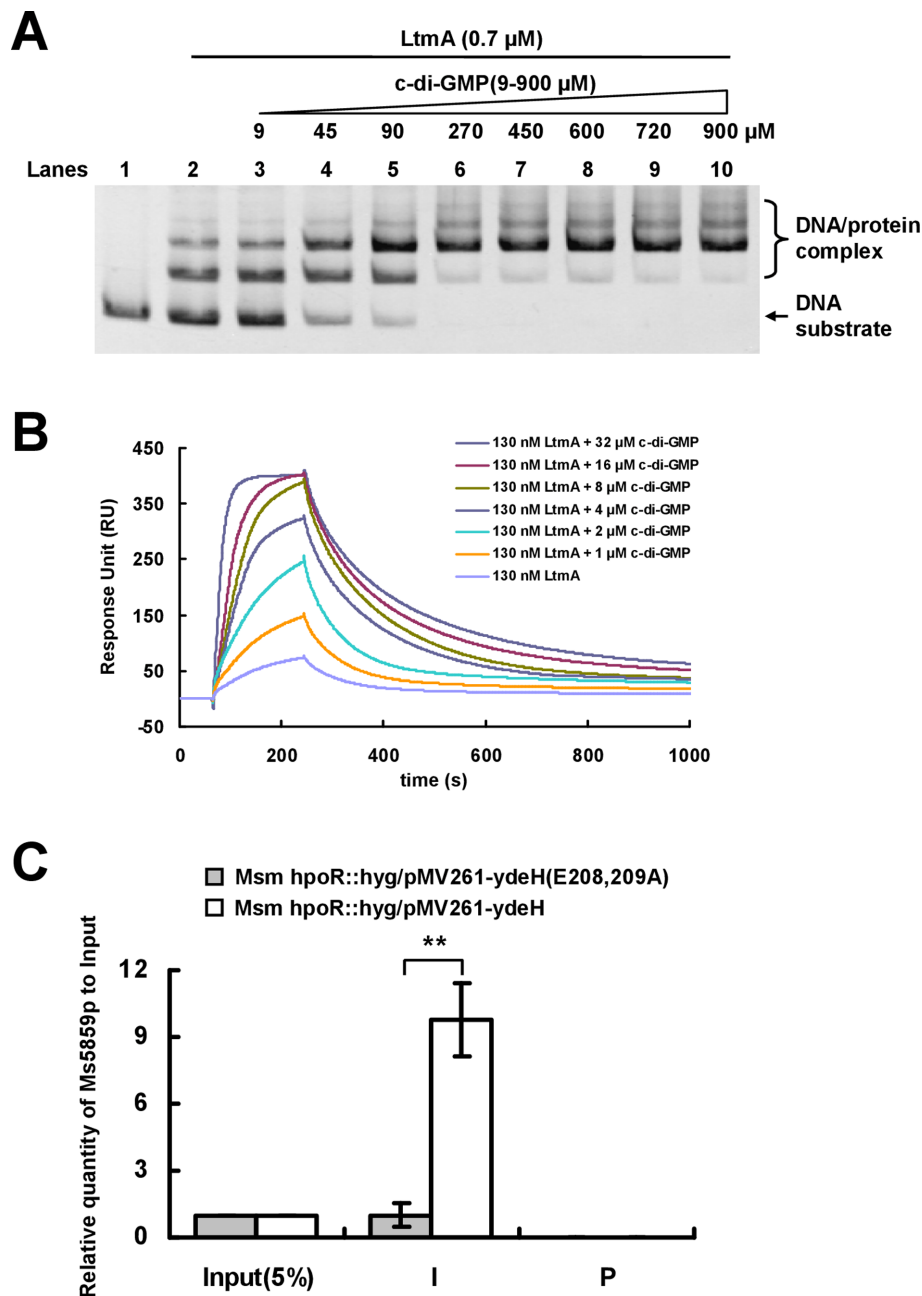
Figure 3. High level of c-di-GMP stimulates the positive regulation of LtmA *in vitro* and *in vivo* in *M. smegmatis*. (**A**) EMSA assays for the stimulating effect of c-di-GMP on the DNA-binding activity of LtmA. Concentration of LtmA was immobilized, and increasing amounts of c-di-GMP (9–900 μM) (lanes 3–10) were added to the reactions and incubated for 30 min. (**B**) SPR assays for the regulatory effect of c-di-GMP on the DNA-binding activity of LtmA. Biotin-labeled Ms5860p was immobilized on the surface of the SA chip, and different concentrations of c-di-GMP were incubated with a fixed concentration of LtmA (130 nM) that was passed over the chip. (**C**) ChIP assays for the effect of c-di-GMP on the intracellular DNA-binding activity of LtmA in *M. smegmatis*. Input (5%) indicated that the supernatant of the disrupted cells was diluted to 5% and used as the template for PCR. ChIP using preimmune (P) or immune (I) sera raised against LtmA. These experiments were quantified using qPCR. The *P*-values of the relative expression data were calculated by two-tailed Student's *t*-test using GraphPad Prism 5. Two asterisks (**) in the figure denote a significant difference ($P \leq 0.01$) between two groups.
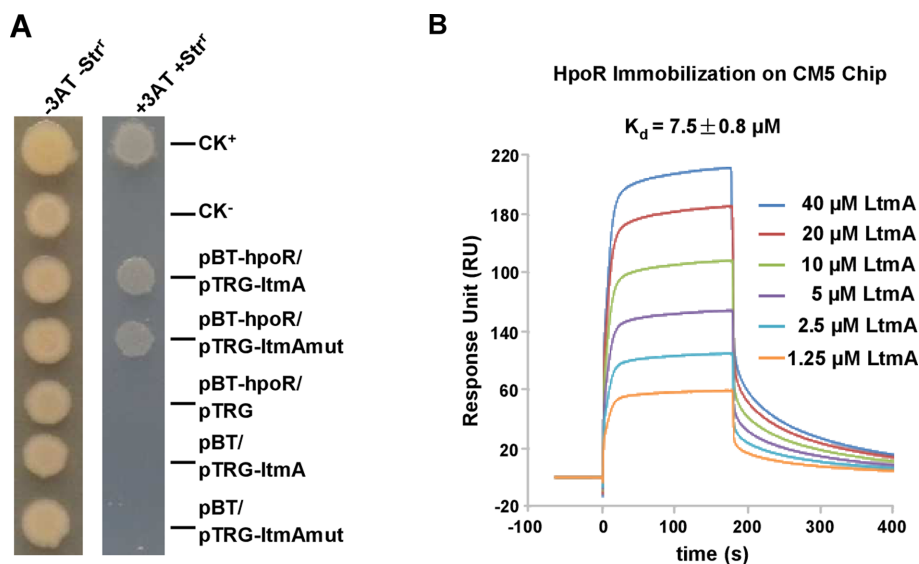
Figure 4. Assays for the interaction between HpoR and LtmA. (**A**) Bacterial two-hybrid assays (Stratagene) for the interaction between HpoR and LtmA. Left panel, plate minus streptomycin (str) and 5 mM 3-amino-1,2,4-triazole (3-AT). Right panel, plate plus str and 5 mM 3-AT. CK$^+$, co-transformant containing pBT-LGF2 and pTRG-Gal11^P was used as a positive control. CK$^-$, co-transformant containing pBT and pTRG was used as a negative control. (**B**) SPR assays for the specific interaction between HpoR and LtmA. HpoR was immobilized on the CM5 chip, and different concentrations of LtmA were passed over the chip.

of a BacterioMatch® II bacterial two-hybrid assay (Stratagene) showed that the reporter strain containing LtmA and HpoR grew well, whereas the control strains containing either LtmA or HpoR alone did not grow on the screening media. This result indicates that LtmA specifically interacts with HpoR. Using purified proteins for a SPR assay, we confirmed the physical interaction between these two regulators. As shown in Figure 4B, the corresponding response increased with increasing amount of LtmA (1.25–40 μM) over the HpoR-immobilized CM5 chip. Kd for the specific interaction between LtmA and HpoR was measured as 7.5 ± 0.8 μM.

Therefore, a physical interaction exists between LtmA and HpoR.

c-di-GMP stimulates the physical interaction between LtmA and HpoR

We further characterized the effect of c-di-GMP on the interaction between LtmA and HpoR. We constructed a LtmA mutant that did not have a c-di-GMP-binding ability but retained a physical interaction with HpoR. Using c-di-GMP-protein cross-linking and LC–MS/MS assays, we successfully mapped out a seven-peptide fragment from LtmA (amino acid residues 114–120), which is responsible for the binding of c-di-GMP (Figure 5A and Supplementary Tables S3 and S4). Thereafter, the mutant protein LtmA△114–120 was produced by deleting the encoding sequence of the seven amino acids (Figure 5B). The mutant protein HpoRmut was produced by deleting the HTH domain (Figure 5C). As expected, LtmA△114–120 lost its c-di-GMP-binding ability (Supplementary Figure S4) but retained its clear interaction with HpoRmut (Figure 5D).

Using a SPR assay, we examined the effect of c-di-GMP on the interaction between LtmA△114–120 and HpoR-

mut. LtmA△114–120 was first immobilized on the surface of the CM5 chip. As shown in Figure 5E, a corresponding increased response was clearly observed when 3.25 μM HpoRmut, which lost DNA-binding activity (Supplementary Figure S5A) but retained c-di-GMP-binding ability (Supplementary Figure S5C), together with an increasing concentration of c-di-GMP (125–2000 μM) was passed over the LtmA-immobilized CM5 chip. These results indicate that c-di-GMP exerts a stimulating effect on the interaction between the two proteins.

Therefore, we characterized a mutant LtmA that lost its c-di-GMP binding ability and utilized it to confirm whether c-di-GMP stimulates the physical interaction between LtmA and HpoR.

HpoR-LtmA interaction enhances the activity of LtmA but neutralizes the inhibition of HpoR

We further examined the regulatory effect of the LtmA–HpoR interaction on their respective DNA-binding activities. To this end, two mutants were further constructed, in which the DNA-binding domain was deleted from their encoding sequence (Figures 5C and 6A). Both mutant proteins, namely, LtmAmut and HpoRmut, did not possess a DNA-binding activity (Supplementary Figure S5A and B) but retained a good interaction with wild-type HpoR or LtmA (Figure 6B) and a c-di-GMP-binding ability (Supplementary Figure S5C). Then, we measured the regulatory roles of the two mutant proteins. As shown in Figure 7A, the addition of increasing amounts of LtmAmut (1.2–6.0 μM) gradually released the shifted DNA substrates by HpoR, and the corresponding unshifted DNA substrates reappeared on the gel (lanes 6–8). By contrast, LtmA obtained a better DNA-binding ability than HpoR, and nearly all DNA substrates were shifted when increasing amounts
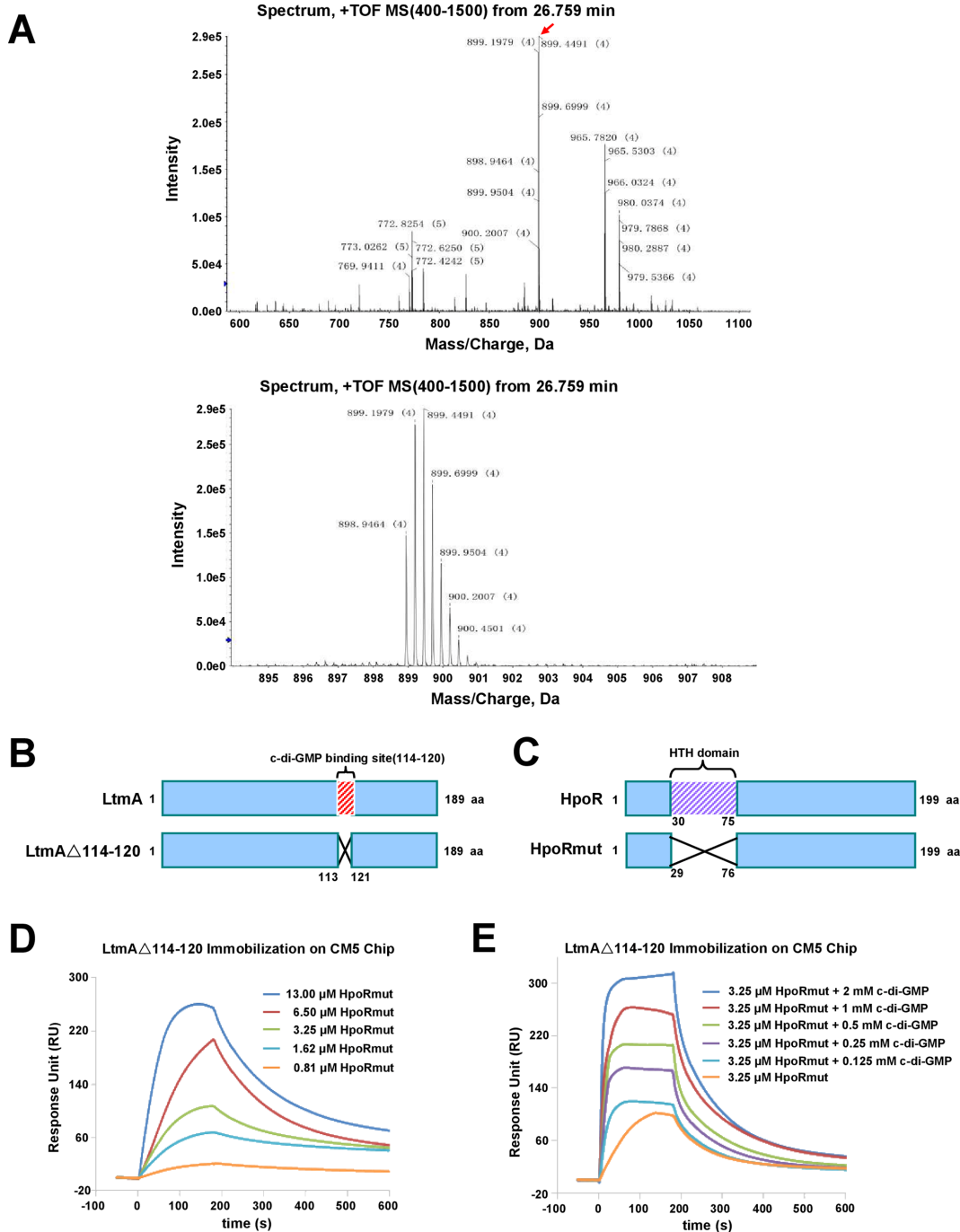
Figure 5. c-di-GMP stimulates the physical interaction between HpoR and LtmA. (**A**) LC–MS/MS assays. Mass identification of a peptide from LtmA cross-linked with a c-di-GMP molecule. LtmA was digested with trypsin, and the products were analyzed by mass. The first-order mass spectrum ($m/\underline{z}$ = 400–1500) is shown (upper panel). The mass spectrum (m/z = 898.94) is indicated by the red arrow, which highly coincides with a potential peptide containing a c-di-GMP molecule in theory (Supplementary Tables S3 and S4). This peptide was further assayed by second-order mass spectrum (lower panel). The charges of the identified peptide were the four charges shown in this figure. (**B**) Schematic of the LtmA mutant protein, LtmA△114–120, which lost the c-di-GMP binding ability. (**C**) Schematic of the HpoR mutant protein, HpoRmut, which lost the DNA-binding activity. (**D**) Assays for the specific interaction between HpoRmut and LtmA△114–120. LtmA△114–120 was immobilized on the CM5 chip, and different concentrations of HpoRmut were passed over the chip. (**E**) SPR assays for the stimulatory effect of c-di-GMP on the interaction between HpoR and LtmA. LtmA△114–120 was immobilized on the surface of the CM5 chip, and different concentrations of c-di-GMP incubated with a fixed concentration of HpoRmut were passed over the chip.
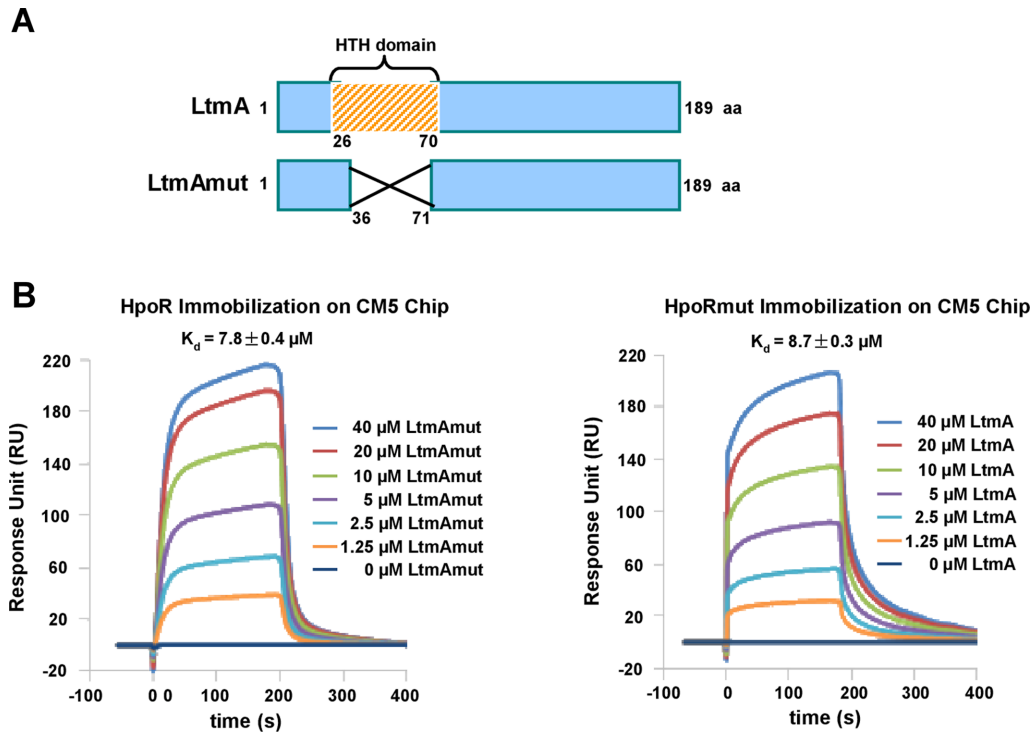
Figure 6. Interaction between a mutant regulator and another wild-type regulator. (**A**) Schematic of the LtmA mutant protein, LtmAmut, which lost the DNA-binding activity. (**B**) SPR assays for the interaction between a mutant regulator and another wild-type regulator. LtmAmut interacted with HpoR (left panel), and HpoRmut interacted with LtmA (right panel).
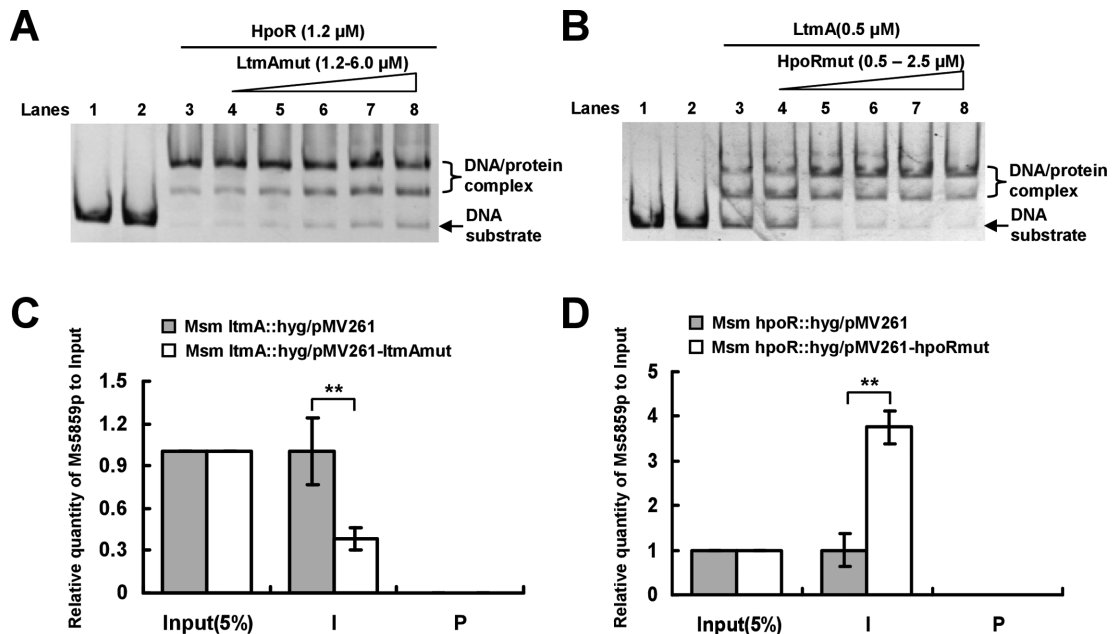
Figure 7. Assays for the regulation of the HpoR-LtmA interaction on the DNA-binding activities of two transcription factors both *in vitro* and *in vivo* in *M. smegmatis*. (**A**) EMSA assays for the inhibitory effect of LtmAmut on the DNA-binding activity of HpoR. (**B**) EMSA assays for the stimulating effect of HpoRmut on the DNA-binding activity of LtmA. (**C** and **D**) ChIP assays for the effect of LtmA or HpoR on the intracellular DNA-binding activity of another regulator in *M. smegmatis*. *ltmA*mut was overexpressed in the *ltmA*-deleted *M. smegmatis* strain for quantifying the precipitated promoter DNA by HpoR upon *ltmA*mut overexpression (C). *hpoR*mut was overexpressed in the *hpoR*-deleted *M. smegmatis* strain. The precipitated promoter DNA by LtmA upon the stimulation of *hpoR*mut overexpression was quantified by qPCR assays (D). Input (5%) indicated that the supernatant of the disrupted cells was diluted to 5% and used as the template for PCR. ChIP using preimmune (P) or immune (I) sera raised against HpoR (C) and LtmA (D), respectively. These experiments were quantified using qPCR. The *P*-values of the relative expression data were calculated by two-tailed Student's *t*-test using GraphPad Prism 5. Two asterisks (**) in the figure denote a significant difference ($P \leq 0.01$) between two groups.

of HpoRmut (0.5–2.5 μM) were added to the reaction (Figure 7B). These results indicate that HpoR stimulates the DNA-binding activity of LtmA, whereas LtmA inhibits the activity of HpoR. Consistently, either hpoRmut or ltmAmut overexpression could enhance the H_2O_2 resistance of *M. smegmatis* (Supplementary Figure S6).

We further assessed the effect of the PPI of LtmA and HpoR on their respective DNA-binding activities *in vivo* in *M. smegmatis*. To suppress the interference of the DNA-binding of LtmA, we overexpressed LtmAmut in the *ltmA*-deleted *M. smegmatis* strain to determine the effect of PPI on the DNA-binding ability of HpoR by using ChIP and qPCR assays. As shown in Figure 7C, HpoR could precipitate ~2.5-fold higher target DNA in the control strain than in the LtmAmut-overexpressed strain, indicating that LtmA could inhibit the DNA-binding ability of HpoR *in vivo* in *M. smegmatis*. This finding is consistent with the *in vitro* results. Using a similar assay by overexpressing HpoRmut in the *hpoR*-deleted *M. smegmatis* strain, we found that HpoR could stimulate the DNA-binding ability of LtmA *in vivo* in *M. smegmatis* (Figure 7D), which is contrary to the effect of LtmA on HpoR.

Therefore, the physical interaction between LtmA and HpoR enhances the activity of LtmA but neutralizes the inhibition of HpoR both *in vitro* and *in vivo* in *M. smegmatis*.

c-di-GMP enhances the stimulation of HpoR on the DNA-binding activity of LtmA

The physical interaction between LtmA and HpoR regulates their respective activities, and c-di-GMP stimulates the interaction, implying that c-di-GMP regulates the effect of one transcription factor on the DNA-binding activity of the other one. To test this hypothesis, we examined whether c-di-GMP can regulate the stimulation of HpoRmut on the activity of LtmA△114–120. As shown in Figure 8A, LtmA△114–120 demonstrated a good DNA-binding ability (lanes 2–4), but c-di-GMP lost its effect on the activity of the mutant protein (lanes 13–17). By contrast, HpoRmut lost its DNA-binding activity (lane 5) but retained its physical interaction with LtmA△114–120 (Figure 5D). The DNA-binding activity of LtmA△114–120 progressively improved when 0.5 μM HpoRmut and an increasing concentration of c-di-GMP (100–400 μM) were added to the reactions (lanes 9–11). These results indicate that c-di-GMP enhances the stimulation of HpoRmut on the DNA-binding activity of LtmA△114–120. Furthermore, the stimulation of c-di-GMP (lanes 9–11) was comparable to the physical interaction of the two proteins on the DNA-binding activity of LtmA△114–120 (lanes 19–21).

Overall, the results indicate that c-di-GMP can enhance the stimulation of HpoR on the DNA-binding activity of LtmA by stimulating the physical interaction between the two regulators.

DISCUSSION

c-di-GMP is an important second messenger in bacteria (1,2) and functions as a global signaling molecule. However, no study has elucidated the mechanism by which c-di-GMP organizes functionally divergent transcription factors

to regulate gene expression for environmental adaptation. To date, no work has reported about the signaling molecule-triggered interaction between its receptor transcription factors in bacteria. In this study, we found that the interaction between LtmA and HpoR is involved in regulating the H_2O_2 resistance of *M. smegmatis*. LtmA functions as a positive regulator and plays an opposing role to HpoR. C-di-GMP can integrate these two functionally opposite regulators into a pathway to drive the expression of antioxidant enzymes. We reported a novel mechanism by which the c-di-GMP signal remodels the transcriptional regulation network for antioxidant defense in mycobacteria.

LtmA is the first characterized c-di-GMP receptor regulator in mycobacteria. It responds to the c-di-GMP signal and positively regulates the expression of antioxidant enzyme genes. By contrast, HpoR functions as a negative regulator that inhibits the expression of detoxification genes (13). Therefore, LtmA plays a functionally opposite regulatory role to HpoR. However, a high-level c-di-GMP clearly attributes the mycobacterial resistance to the stress, implying that a c-di-GMP-integrated mechanism resolves the conflict between these two divergent regulators. In this study, we showed that LtmA could physically interact with HpoR. High-level c-di-GMP stimulates the positive regulation of LtmA and neutralizes the inhibitory role of HpoR. It also boosts the physical interaction between these two regulators, thereby further enhancing the DNA-binding ability of LtmA and reducing the inhibitory activity of HpoR. To our knowledge, this work is the first to report on the mechanism by which c-di-GMP organizes functionally opposite regulators to coordinately drive gene expression for coping with environmental stress. However, obvious challenges exist in addressing the complex interaction among c-di-GMP, two regulators, and the target DNA. Fortunately, we successfully produced their respective DNA-binding mutant proteins. A short peptide within LtmA was found essential for c-di-GMP recognition and the basic amino acid Lys120 within the short peptide was further shown to play an important role for the binding of c-di-GMP with LtmA (Supplementary Figure S7). Basing on these mutant proteins, we uncovered a novel c-di-GMP-triggered regulation mechanism for oxidative stress defense in mycobacteria. This finding would provide important insights into the regulatory network of c-di-GMP and its correlation with bacterial adaptation to environmental stress.

Although a similar regulatory sequence was recognized within the upstream fragment of the *hpoR* operon, LtmA and HpoR exerted unexpectedly opposite effects on the gene cluster expression and bacterial H_2O_2 resistance. In particular, their antiserums had cross reactions with the two regulators (data not shown), thereby preventing the use of a traditional pull-down assay to evaluate the interaction between the two regulators. This finding is consistent with the fact that the two proteins had a 43% amino acid sequence identity (Supplementary Figure S1). The functional divergence was probably due to their different aggregation states because more slow-shifted DNA–protein complex bands could be observed for LtmA (Figure 3A) at high protein concentrations. However, the underlying molecular mechanism for the divergences between two regulators has yet to be elucidated. In addition, the DNA-binding affinity of
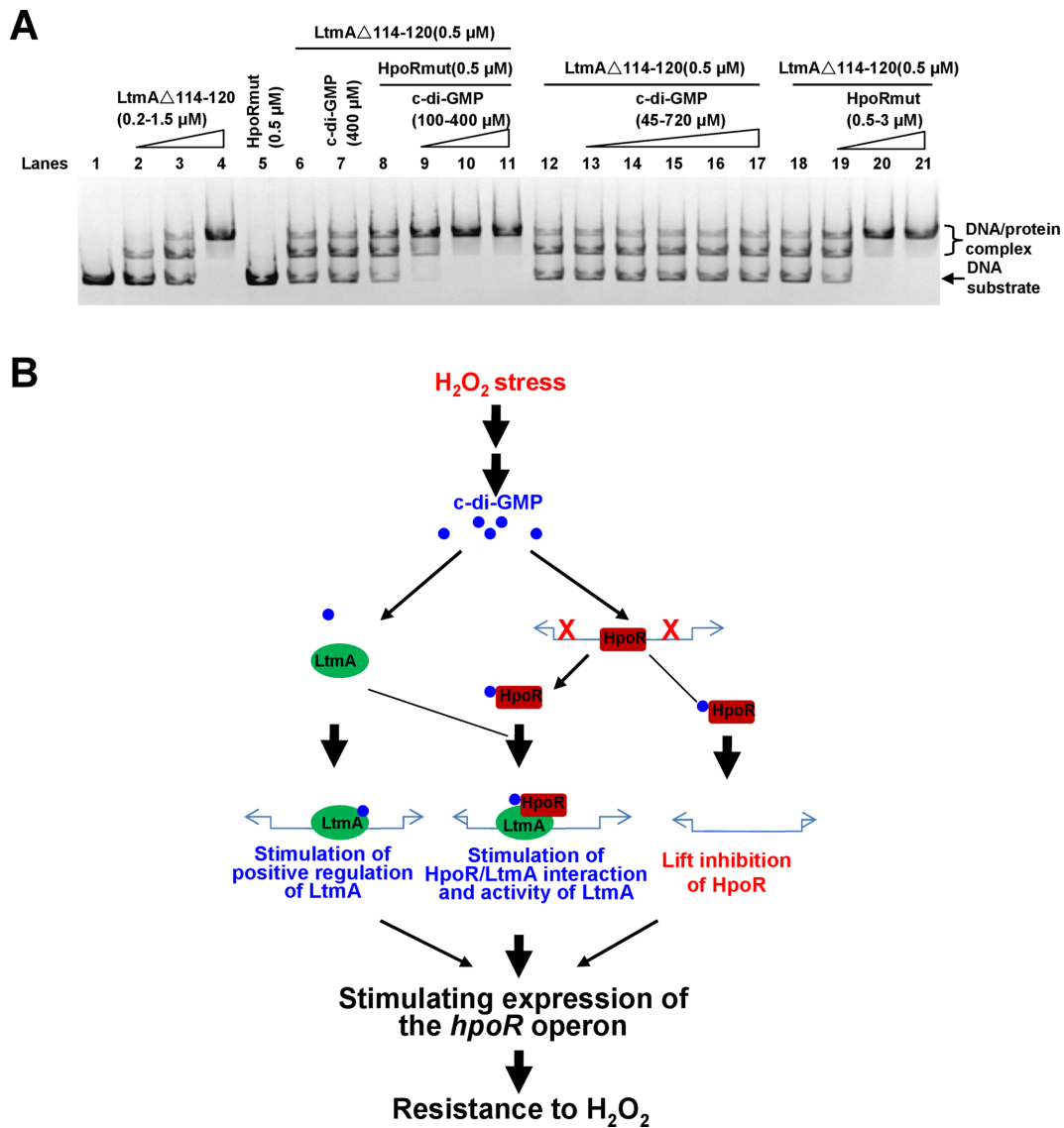
Figure 8. EMSA assays and the antioxidant model. (**A**) c-di-GMP enhanced the stimulation of HpoR on the DNA-binding activity of LtmA. HpoRmut lost the DNA binding activity (lane 5) but retained the c-di-GMP binding ability. By contrast, LtmA△114–120 lost the c-di-GMP binding activity and retained the DNA binding activity (lanes 1–4). c-di-GMP alone did not stimulate the DNA-binding activity of LtmA△114–120 (lanes 13–17), but c-di-GMP obviously stimulated the activity in the presence of 0.5 μM HpoRmut (lanes 9–11). An increasing concentration of HpoRmut stimulated the DNA-binding activity of LtmA△114–120 (lanes 19–21). (**B**) A model showing that c-di-GMP integrates the two regulators into a pathway to drive the expressions of antioxidant enzymes in three ways.

these two receptor transcription factors to the DNA sequence also displayed an obvious difference (Supplementary Figure S8). The Kd for HpoR (0.29 μM) was approximately twofold lower than that of LtmA (0.62 μM), suggesting that HpoR possesses a stronger competitive DNA-binding ability in the absence of, or at a low level of, c-di-GMP. This phenomenon would repress the additional expression of antioxidant enzymes in the absence of stressful conditions. However, upon oxidative stress, a high level of c-di-GMP accumulated in the bacterial cells (13), which enhanced the affinity of LtmA to DNA but inhibited the activity of HpoR, thereby reversing the competitive ability of the two regulators for the target DNA and triggering the expression of the *hpoR* operon for antioxidant defense. There-

fore, these characteristics of the two transcription factors and their regulation of the *hpoR* operon are beneficial to the bacterial adaptation to stressful environments.

In summary, the present study, together with our previous studies (13,27), revealed that c-di-GMP could integrate two divergent regulators to trigger the gene expression in three ways, as shown in Figure 8B. First, c-di-GMP can stimulate the positive effect of LtmA on the expression of the *hpoR* operon to enhance the mycobacterial H_2O_2 resistance. Second, c-di-GMP can neutralize the inhibition of HpoR on the *hpoR* operon. Third, c-di-GMP can boost the interaction between LtmA and HpoR to further enhance the positive regulation of LtmA and inhibit the negative regulation of HpoR. These findings provide new insights into

the mechanism by which c-di-GMP remodels the transcriptional regulatory network to protect bacteria against environmental stress.

DATA AVAILABILITY

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

FUNDING

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