A Copper-responsive Global Repressor Regulates Expression of Diverse Membrane-associated Transporters and Bacterial Drug Resistance in Mycobacteria

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Background: The regulators involved in the expression of bacterial multidrug resistance transport proteins remain largely unknown. Although the regulators and signaling pathways involved in the expression of these genes remain largely unknown, the regulators and signaling pathways involved in the expression of these genes remain largely unknown. In this study, we have identified Ms2173, a GntR/Fad family transcription factor, as a novel global regulator in Mycobacterium smegmatis. Ms2173 was found to specifically recognize a 15-bp palindromic motif and to broadly regulate expression of 292 genes, including 37 genes that encode membrane-associated transport proteins. Copper ions induced Ms2173 to form inactive proteins lacking DNA-binding activity. Ms2173 was shown to function as a repressor of its target genes. Interestingly, we found that the function of Ms2173 was linked to mycobacterial drug resistance. Compared with the substantially enhanced drug resistance in the Ms2173-deleted mutant strain, the strains overexpressing Ms2173 were more sensitive to anti-tuberculosis drugs than the wild-type strain. Additionally, copper ions could partially counteract the in vivo function of Ms2173. We have thus characterized the first mycobacterial GntR/Fad family transcription factor that functions as a copper ion-responsive global repressor that we have renamed GfcR. These findings further enhance our understanding of membrane-associated transporter regulation and drug resistance in mycobacteria.

In recent decades, the appearance and spread of bacterial drug resistance has become a major health issue worldwide.

Important pathogens such as Mycobacterium tuberculosis, the causative microbe for tuberculosis, have now acquired multidrug resistance (MDR)³. Understanding the regulatory mechanism of bacterial drug resistance is therefore an important and urgent goal with significant implications for human health and disease worldwide.

Several studies have shown that bacterial drug resistance occurs by numerous mechanisms, including enzymatic inactivation, drug target alteration or protection, and reduced uptake (2, 3). Among them, increase in active membrane-bound efflux pumps that transport toxic antibacterial drugs from the cell is a major concern, especially because individual MDR pumps are capable of exporting a number of structurally dissimilar drugs (4, 5). In recent years, a number of bacterial regulatory proteins that govern the expression of drug transporters have been characterized (3). Such proteins encompass both repressors and activators such as the Escherichia coli TetR and EmrR (6, 7), the Staphylococcus aureus LacR (8), and the Bacillus subtilis BmrR (9), which inhibit or stimulate the expression of their target efflux genes. Interestingly, several global regulators such as MarA, Rob, and SoxS have been shown to enhance the expression of the E. coli acrAB MDR locus (10). These studies have provided a basic picture of the regulatory pathways controlling the expression of drug efflux genes in bacteria. However, additional MDR transporter homologs have been identified by sequencing the entire bacterial genome, and their regulators and underlying regulatory mechanisms remain to be explored (11).

Mycobacterium smegmatis is a fast-growing model mycobacterium and has been widely used to study the gene regulatory mechanism of virulent and slow-growing species like M. tuberculosis (12, 13). The genomes of both M. smegmatis (GenBank™ ACCESSION NUMBER CP000480) and M. tuberculosis encode at least two dozen putative drug efflux transporters (14).

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Several of these transporters have been shown to contribute to mycobacterial resistance to isoniazid (INH), rifampicin (RIF), tetracycline, and other toxic compounds (15–17). Strikingly, M. smegmatis contains more than 500 potential regulatory factors. The large number of putative drug efflux systems and their potential regulators, therefore, underscore the complexity of the mechanisms involved in regulation of drug resistance in M. smegmatis (18). However, potential regulators involved in broad regulation of expression of membrane-associated transporter genes have not been successfully isolated to date.

GntR, named after a repressor of the B. subtilis glucosylate operon (19), is a large, poorly characterized transcriptional regulation family both in M. smegmatis and M. tuberculosis (20, 21). GntR family proteins possess a highly conserved N-terminal winged helix-turn-helix domain for DNA-binding and a diverse C-terminal ligand-binding domain for effector-binding/oligomerization (19). The variable C-terminal domain provides a basis for their classification into subfamilies such as FadR, HutC, MocR, YtrA, AraR, and PlmA (22). FadR is the largest subfamily, comprising ~40% of all GntRs, and most of them are involved in the regulation of oxidized substrates such as pyruvate (PdhR), glucosylate (GntR), glycolate (GlcC), and L-lactate (LldR) (22–25). FadR binds DNA as a homodimer and functions as a repressor of the fad regulon in E. coli (26–29). However, no GntR/Fad family regulator has been functionally identified and characterized in mycobacteria to date.

In this study, we have successfully isolated and characterized the first GntR/FadR-like transcription factor, Ms2173, in M. smegmatis. Ms2173 acts as a novel master regulator that responds to copper ions and regulates expression of a diverse set of genes that includes 37 membrane-associated transporters. Furthermore, we provide evidence to show that Ms2173 functions as a global repressor and negatively affects mycobacterial drug resistance. Thus, we have identified a new regulatory pathway for bacterial drug resistance that is mediated by a unique copper ion-responsive GntR/FadR-like regulator in mycobacteria.

**EXPERIMENTAL PROCEDURES**

**Strains, Enzymes, Plasmids, and Reagents—**E. coli BL21 strains and the pET28a plasmid were purchased from Novagen. All restriction enzymes, T4 ligase, Pyrobest DNA polymerase, strains and the pET28a plasmid were purchased from Novagen. INH. The used INH concentration (10 μg/mL) was shown according to multiple experiments in which condition the INH-sensitivities of transformants were easily observed.

The recombinant plasmids were isolated from the INH-sensitive M. smegmatis transformants and, therefore, the negative regulator genes could be characterized.

**Expression and Purification of Recombinant Protein and PAGE Analysis—**Ms2173 and its mutant genes were amplified by PCR from the genomic DNA of M. smegmatis mc²155 and were cloned into the pET28a vector to produce recombinant vectors. After being transformed with the recombinant plasmid (supplemental Table S5), E. coli BL21 cells were grown in 200 ml Luria Bertani medium up to an A₆₀₀ of 0.6. Protein expression was induced by the addition of 0.5 mm isopropyl β-D-1-thiogalactopyranoside. The cells were collected, and proteins were purified using an affinity column as described previously (31). The elution was dialyzed overnight and stored at ~80 °C. A common SDS-PAGE was used to determine the protein molecular weight.

**Homology Structure Modeling—**The structure of Ms2173 was modeled computationally using the automated comparative protein modeling web server SWISS-MODEL (32). The E. coli FadR family of proteins (27) was used as a template (PDB code 1E2X).

**DNA Substrate Preparation and EMSA—**DNA fragments for the DNA-binding activity assays were amplified by PCR from M. smegmatis mc²155 genomic DNA or directly synthesized by Invitrogen (supplemental Table S5). The DNA substrates were labeled and prepared as described previously (31) and stored at ~20 °C until use. EMSA experiments with labeled DNA fragments were performed as described previously (12). Images were acquired using a Typhoon scanner (GE Healthcare).

**DNase I Footprinting Assays—**The 150-bp promoter regions of the Ms2173 gene (coding strand and non-coding strand) (supplemental Table S3) were amplified by PCR using appropriate primers labeled with fluorescein isothiocyanate (supplemental Tables S1 and S3). The amplified products were purified with a DNA purification kit (BioFlux) and then subjected to similar binding reaction as in EMSA. DNaseI footprinting was performed as described previously (13). The ladders were produced using the Sanger dyeoxy method.

**Construction of the Ms2173 Deletion Mutant of M. smegmatis and Southern Blot Analysis—**Knockout of the Ms2173 gene from M. smegmatis was performed as described previously (31, 33) with modifications. The recombinant plasmid pMinds-Ms2173 was constructed and electroporated into M. smegmatis. Allelic exchange mutants in which the Ms2173 gene had been deleted were identified by restriction digestion and subsequent PCR analysis using the primers on each side of Ms2173 and the hygromycin gene. Deletion of Ms2173 was further verified by a previously described Southern blot analysis procedure (31). Genomic DNA was digested overnight with an excess of BsrI, and the fragments were separated by electrophoresis through 1.0% agarose gels. The probe consisted of a 239-bp fragment from the downstream region of the Ms2173 gene amplified using appropriate primers (supplemental Table S1).

**Quantitative Real-time PCR—**Isolation of mRNA and cDNA preparation of the MsmpMindD and MsmpMmindD-Ms2173 (Msmp and MsmpMs2173::hyg) strains was performed, and real-time PCR analysis was subsequently carried out according to procedures described previously (31). The reac-
Characterizations were performed in a Bio-Rad IQ5 RT-PCR machine. Amplification specificity was assessed using a melting curve analysis. Gene expression levels were normalized to the levels of 16S rRNA gene transcripts. The degrees of expression change were calculated using the $2^{-\Delta\Delta C_t}$ method (31, 34). Average relative expression levels and standard deviations were determined from three independent experiments.

Chromatin Immunoprecipitation Assay—ChIP was performed as described previously (31). Briefly, exponentially growing M. smegmatis cells were fixed with 1% formaldehyde, and fixation was stopped by adding 0.125 M glycine for 5 min. Cross-linked cells were harvested and resuspended. Samples of those cells were sonicated on ice and incubated with 10 μl of antibodies against Ms2173 or preimmune serum under rotation for 3 h at 4 °C. The complexes were immunoprecipitated with protein A-agarose for 1 h. Cross-linking was reversed for 6 h at 65 °C. DNA samples of the input and the immunoprecipitates were purified and analyzed by PCR using Platinum Taq (Invitrogen). The amplification protocol included one denaturation step of 5 min at 95 °C and then 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C.

Determination of Mycobacterial Growth Curves and the Effect of Drugs and Metal Ions—Growth patterns of the wild-type (Msm), Ms2173-deleted mutant (Msm/ΔMs2173), and overexpression (Msm/pMindD-Ms2173) mycobacterial strains were determined according to procedures described previously (12) with some modifications. M. smegmatis was grown in 100 ml of 7H9 medium when needed, and 10 μg/ml kanamycin and 25 ng/ml Tet or 30 μg/ml INH were added. Each mycobacterial strain was also freshly grown in Middlebrook 7H9 medium containing 50 μg/ml hygromycin and 10 μg/ml kanamycin if needed. When cells reached a stationary growth phase with an A$_{600}$ of 1.5 to 2.0, the cultures were diluted in Middlebrook 7H9 medium to an A$_{600}$ of 0.2 and split for induction with or without 2.5 μM metal ions (Cu$^{2+}$ or Zn$^{2+}$) or drugs (30 μg/ml INH or 2 μg/ml Rif). The cultures were conducted with an additional growth in 7H10 medium at 37 °C at 200 rpm for 3–4 days. Aliquots were taken at the indicated times, and A$_{600}$ was measured. At the same time, a small aliquot of the culture was plated on 7H10 medium for determining colony forming units.

RESULTS

M. smegmatis Ms2173 Potentially Contributes to Mycobacterial INH Sensitivity—To identify potential transcription factors that regulate drug resistance in M. smegmatis, we screened the transcriptional regulator library by spotting these recombinant strains on plates containing INH (10 μg/ml). A hypothetical transcription factor named Ms2173 was isolated as a potential contributor to INH sensitivity in M. smegmatis. As shown in Fig. 1A, the mycobacterial strain transformed with pMind-Ds2173 and, thus overexpressing Ms2173, grew very slowly on the screening plate and was more sensitive to 10 μg/ml of INH than the wild-type strain. In contrast, recombinant strains overexpressing two unrelated genes, Ms0895 and Ms1117, under the same condition had similar growth as the wild-type strain (Fig. 1A). This suggests that Ms2173 is potentially involved in regulating INH drug resistance in M. smegmatis. A further sequence BLAST assay found that Ms2173 contained an N-terminal winged helix-turn-helix DNA-binding domain and a typical FadR-like C-terminal domain, indicating that Ms2173 encodes a GntR/FadR-like regulator (Fig. 1B).

Copper Ion Specifically Induces Ms2173 to Form an Inactive DNA-binding Protein—We measured the regulatory effects of several metal ions on the DNA-binding activity of Ms2173 (supplemental Fig. S1), and only copper ions (Cu$^{2+}$) could inhibit the DNA-binding activity of Ms2173. We further examined the Cu$^{2+}$ concentration-dependent inhibition on the DNA-binding activity of Ms2173. As shown in Fig. 2A, 6 μM of the Ms2173 protein alone could shift the mobility of DNA substrates in the reactions. However, a stepwise decrease in the formation of DNA/protein complexes was clearly observed with the addition of increasing amounts of Cu$^{2+}$ into the reactions (Fig. 2A). This indicates that the DNA-binding activity of Ms2173 is inhibited by Cu$^{2+}$. In contrast, zinc ions had no similar role under the same conditions (Fig. 2B).

Alternatively, when Ms2173 was first preincubated with DNA substrates for 30 min and the same amount of Cu$^{2+}$ as above was then added into the reactions, the inhibitory effect of Cu$^{2+}$ was significantly lower (Fig. 2A, right panel). This indicates that the binding affinity of Cu$^{2+}$ to Ms2173 is weak compared with that of Ms2173 to DNA.

Ms2173 Binds Target DNA Fragments Containing a Palindromic Sequence Motif—Several truncated DNA substrates covering the promoter region of Ms2173, designated as S1–S3 (Fig. 3A), were produced to characterize the DNA motif recognized by the Ms2173 protein. An obvious DNA-binding activity was observed with the 67-bp substrate S1, but not with S2 or S3.
Ms2173 broadly Recognizes the Promoters of Diverse Genes, Including 37 Membrane-associated Transporter Genes—A total of 292 potential target genes were identified when the intergenic regions of the \textit{M. smegmatis} genome were searched on the basis of the sequence motif identified above (supplemental Table S6). We then analyzed the classification and number of these target genes in the context of clusters of orthologous groups (COG) categories. As shown in Fig. 5, A and B, among several defined COG categories, strikingly, the potential targets included promoters of 37 membrane-associated transporter genes (supplemental Table S7). Using the WebLogo tool (35), a more general conserved motif sequence for Ms2173 binding was characterized, as shown in Fig. 5C. Furthermore, target promoters of all 37 of these genes could be specifically recovered by chromatin immunoprecipitation using the Ms2173 antibody (Fig. 5D). Importantly, the Ms2173 antibody could not recover Ms0103p, a negative control. Further EMSA experiments also confirmed that Ms2173 could bind with many upstream DNA regions of these target genes (supplemental Fig. S2). These results indicate that Ms2173 can broadly recognize the promoters of a large number of membrane-associated transporter genes.

Ms2173 Negatively Regulates the Expression of Many Membrane-associated Transporter Genes—To characterize the function of Ms2173, an Ms2173-deleted mutant strain of \textit{M. smegmatis} was generated by a gene replacement strategy (supplemental Figs. S3 and S4). We then compared the expression of some membrane-associated transporter genes in both wild-type and Ms2173-deleted mutant \textit{M. smegmatis} strains using quantitative real-time PCR assays. As shown in Fig. 6A, expressions of most of the tested genes were significantly up-regulated ($p < 0.05$) in the mutant strains compared with that in the wild-type strain. However, the expression level of the negative control gene MsSigA, which lacked the conserved motif in its promoter, did not change obviously. This finding suggested to us that Ms2173 could function as a negative regulator in \textit{M. smegmatis}. Further overexpression experiments also confirmed this observation. As shown in Fig. 6B, expressions of the tested target genes were significantly down-regulated ($p < 0.05$) when Ms2173 was overexpressed (about 5-fold) through a pMind-derived recombinant plasmid in \textit{M. smegmatis}.

Next, we compared relative gene expression levels in response to different metal ions in the Ms2173-overexpressed strains. As shown in Fig. 6C, expressions of the tested target genes were significantly up-regulated ($p < 0.05$) in response to Cu$^{2+}$ but not to Zn$^{2+}$. This result is similar (albeit weaker) to that observed in the Ms2173-deleted mutant \textit{M. smegmatis} strain shown above (Fig. 6A), suggesting that Cu$^{2+}$, but not Zn$^{2+}$, could counteract the repressive regulation of target genes by Ms2173. Taken together, our results indicate that Ms2173 negatively regulates the expression of its target genes and that this function of Ms2173 is sensitive to Cu$^{2+}$.

Ms2173 Negatively Regulates Drug Resistance in \textit{M. smegmatis}—We determined mycobacterial growth curves to examine the regulatory effect of Ms2173 on the growth of \textit{M. smegmatis} in response to INH and RIF. As shown in Fig. 7A, compared with the wild-type strain with a minimal inhibition
concentration value of 200 μg/ml, the Ms2173-overexpressed M. smegmatis strain grew extremely slowly in the Middlebrook 7H9 medium containing 30 μg/ml INH. Conversely, the Ms2173-deleted mutant exhibited faster growth compared with the wild-type strain under the same conditions (Fig. 7B). When expressing the Ms2173 gene through a pMind plasmid in the Ms2173-deleted mutant M. smegmatis, the recombinant strain reobtained a slow growth in the Middlebrook 7H9 medium containing 30 μg/ml INH (supplemental Fig. S5), indicating that the growth differences between Ms2173-deleted mutant and the wild-type strain were due to loss of Ms2173. Similarly, Ms2173-overexpressed M. smegmatis strains grew slowly in the 7H9 medium containing 2 μg/ml RIF. However, growth of the Ms2173-deleted mutant strain was comparable with that of the wild-type strain at this concentration of RIF (supplemental Fig. S6). No obvious growth difference between the wild-type and recombinant strains was observed when the drugs were removed from the medium (supplemental Fig. S6).

We further determined the lowest concentration of INH (MIC value) resulting in complete inhibition of growth or in growth of ≤ 1% of the initial inoculum of different mycobacterial strains. Compared with the wild-type strain with a MIC value of 200 μg/ml, the Ms2173-overexpressed M. smegmatis strain had a lower MIC value of 160 μg/ml. Conversely, the Ms2173-deleted mutant had a higher MIC value of 300 μg/ml.

These results indicate that Ms2173 negatively regulates mycobacterial INH and RIF resistances. Taken together, our observations support a model in which Ms2173 represses the expression of many membrane-associated drug efflux pumps and thus inhibits the bacterial ability to get rid of drugs from the cell (Figs. 5 and 6).

Cu²⁺ Can Partially Reverse the Function of Ms2173 in Vivo in M. smegmatis—We have shown that Cu²⁺ can inhibit the ability of Ms2173 to bind DNA (Fig. 2) and to negatively regulate the expression of its target genes (Fig. 6). If the above-mentioned growth phenotypic changes are mediated by the DNA-binding ability and transcriptional regulatory activity of Ms2173, Cu²⁺ should reverse or at least decrease the severity of these phenotypes. To test this possibility, we examined the effect of Cu²⁺ on the growth of the Ms2173-overexpressed M. smegmatis strain in the presence of INH. As shown in Fig. 7C, 2.5 μM Cu²⁺ could strongly reduce the inhibition of 30 μg/ml INH on the growth of the overexpression strain (Fig. 7A), although Cu²⁺ had a certain toxicity to the mycobacterial growth (supplemental Fig. S7). This effect was specific to Cu²⁺, as addition of Zn²⁺ did not produce the same result (Fig. 7C). In addition, when INH was removed from the medium, no obvious difference could be observed in the growth of the overexpression strain in response to the different ions (Fig. 7D and supplemental Fig. S8).

DISCUSSION

Membrane-associated transport proteins play important roles in bacterial adaptation to environmental stresses, includ-
ing antibacterial drugs. With recent success in sequencing bacterial genomes, many of these transporters, including the MDR transport proteins, have been identified. However, the regulators and regulatory mechanisms involved in the expression of most of these genes remain largely unknown. In this study, we have identified Ms2173, a GntR/FadR family transcription factor, as a novel global transcriptional regulator in *M. smegmatis*. We further uncovered that Ms2173 underlies a new Cu^{2+}/H{sup+}-responsive pathway for the regulation of mycobacterial membrane-associated transporter expression and, thereby, the regulation of drug resistance in *M. smegmatis*.

The genome of the fast-growing bacterium *M. smegmatis* encodes more than 500 potential transcriptional regulation genes. Interestingly, several broad regulators have been characterized in *M. smegmatis* and other bacterial species (36). In a recent study, Yang et al. (37) found that *M. smegmatis* Ms6564, a TetR-like transcription factor, is involved in regulating the expression of a large number of DNA repair/damage genes. In this study, *M. smegmatis* Ms2173, a GntR/FadR-like protein, was confirmed as a candidate for broadly regulating the expression of membrane transport genes and other genes with diverse functions. Regulators of the GntR/FadR family are widely dis-
distributed among bacteria (22), and they are known to be involved in the regulation of multidrug resistance, biosynthesis of antibiotics, osmotic stress, and virulence (38, 39). In this study, using DNaseI footprinting and EMSA assays, a 15-bp palindromic sequence required for specific recognition by Ms2173 was identified. Furthermore, we identified the conserved binding motif for Ms2173 within the promoters of 292 M. smegmatis genes or operons. These potential target genes covered a variety of gene families, including membrane transport genes, transcriptional regulators, acyl-coA-related genes, and many metabolism genes (Fig. 5 and supplemental Table S6). On the basis of this result, we conclude that Ms2173 functions as a broad transcriptional regulator in M. smegmatis. Interestingly, an ortholog of Ms2173, Rv0494, was also found in the pathogen M. tuberculosis H37Rv (supplemental Fig. S9).

Bacterial drug efflux pumps have been categorized into five families, including the ATP-binding cassette superfamily, the major facilitator superfamily, the multidrug and toxic com-
pound extrusion family, the small multidrug resistance family, and the resistance-nodulation-division superfamily (3). These transporters export a wide array of substrates from the inside to the outside of the cell and contribute to bacterial drug resistance (40, 41). In this study, Ms2173 was found to regulate the expression of 37 potential membrane-associated transporter genes. Some of these target genes belong to several superfamilies of bacterial drug efflux pumps. For example, 13 of the 37 genes are hypothetical proteins that belong to the ATP-binding cassette superfamily. Four proteins encoded by Ms0716, Ms1329, Ms3316, and Ms6375 belong to the major facilitator superfamily (supplemental Table S7). In addition, a drug resistance transporter of the Bcr/CfiA superfamily, encoded by Ms5047, was also one of the targets identified in our study (supplemental Table S7). Interestingly, the list of target genes also included Ms0695, which encodes an isoniazid inducible pro-

**FIGURE 6.** Differential expression assays of several membrane-associated transporter genes in the wild-type, mutant, and Ms2173-overexpressed strains. The mycobacterial cDNA was amplified, and quantitative real-time PCR assays were performed as described under “Experimental Procedures.” Relative expression levels of the genes were normalized using the 16S rRNA gene as an invariant transcript, and an unrelated sigA gene was used as a negative control. Relative expression levels of target genes in Msm and Msm/ΔMs2173 (A), in Msm/pMindD and Msm/pMindD-Ms2173 (B), and in Msm/pMindD-Ms2173 strains in response to different metal ions (C) were assayed. As a positive control, total DNA of each strain was used as template for PCR amplification. The cDNA of the mutant strains and the recombinant strain containing an empty pMindD vector was used as template in the negative controls. Data were analyzed using the 2-fold method (30). Relative expression data were analyzed for statistical significance by the unpaired two-tailed Student’s t test using GraphPad Prism. *, p ≤ 0.05.
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The operon iniBAC, covering the iniA gene, was shown previously to confer multidrug resistance to Mycobacterium bovis Bacille de Calmette Guerin through an associated pump-like activity (42, 43). A recent study further found that the operon iniBAC is regulated by a two-component regulator, MtrA, which is involved in drug resistance in M. smegmatis (13). Our findings thus support our model that Ms2173 is involved in global regulation of mycobacterial drug resistance.

Many bacterial regulators have been characterized previously to regulate individual operons involved in drug resistance (3). Very few broad regulators and their associated target operons have been characterized so far. MarA, as well as its homologs, is one of the few examples of genes that have been characterized as a global regulator. MarA is known to function as an activator, and only some of their targets have been characterized clearly (3). In this study, we found that Ms2173 regulates multiple drug resistance-associated genes. Furthermore, by constructing recombinant strains overexpressing Ms2173 and Ms2173-deleted mutant strains and analyzing the expression of target genes in these strains by quantitative real-time PCR assays, we found that, unlike MarA, Ms2173 acts as a repressor for the expression of its targets. Our results support a model in which Ms2173 prevents constitutive expression of these membrane transporters, thereby conferring good drug responsiveness to wild-type M. smegmatis strains. Consistent with this, we observed that the Ms2173-overexpressed strains were more sensitive to the drug than the wild-type strains (Fig. 7). In contrast, the Ms2173 deletion strains displayed much stronger drug resistance than the wild-type strain (Fig. 6). Our results show that Ms2173 is a global repressor that negatively regulates the expression of multiple drug efflux pumps in M. smegmatis.

Metal ions, including copper, iron, and zinc, have important roles in all living cells. In intracellular pathogens such as M. tuberculosis, inorganic ions have been shown to be involved in regulating the expression or activity of some important proteins (44). For example, copper is essential for the ability of the protein SodC to control an intracellular oxidative burst during macrophage survival (45). Three major prokaryotic copper metalloregulators (CopY from Enterococcus hirae, CueR from E. coli, and B. subtilis, and CsoR from M. tuberculosis) have been identified and characterized in bacteria (46–49). Until now, only a FadR-like regulator, TM0439, has been found to bind metal ions (Zn$^{2+}$) (50). However, the regulatory mechanism and functional effects of the metal ions on the regulator remains unclear. Recently, a copper-responsive transcription factor, RicR, was found to function as a global regulator in M. tuberculosis (51), which is a dimer in solution and belongs to the CsoR family (49). However, Ms2173 had no sequence similarity to the RicR in M. tuberculosis (data not shown). In this study, Ms2173 was characterized as a Cu$^{2+}$-responsive regulator. We confirmed that Cu$^{2+}$ could specifically counteract the repressive effects of Ms2173 on the expression of its target genes and on bacterial drug resistance. These findings indicate that Ms2173 is different from RicR and other FadR proteins (27, 49, 50) and is a new copper ion-responsive transcription factor. Interestingly, the potential target genes of Ms2173 include a copper resistance protein, CopC (Ms6436), and a metal ion channel membrane protein (Ms1945). This implies that Ms2173 could be involved in regulation of the intracellular concentration of copper ions in M. smegmatis. Taken together, copper ions may cross-talk with Ms2173, which regulates the activity of multiple drug efflux pumps and, therefore, affect the drug resistance of M. smegmatis.

In summary, a new transcription factor belonging to the GntR/FadR-like family was successfully isolated and characterized in M. smegmatis. The promoters of about 292 M. smegmatis genes or operons were characterized as its potential targets. Notably, Ms2173 was found to be involved in the regulation of the expressions of 37 membrane transport genes and to negatively affect mycobacterial drug resistance. Copper ions could inhibit the DNA-binding activity of Ms2173 in vitro and partially counteract its in vivo functions. Our findings establish Ms2173 as a novel copper ion-responsive global repressor in mycobacteria and significantly enhance our understanding of the regulatory mechanism and signaling pathway associated with bacterial drug resistance.

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