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Research Article 研究报告

A TetR-like regulator negatively regulates isoniazid resistance in *Mycobacterium smegmatis*

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Abstract: [Objective] To study the regulatory effect of TetR family transcription factor Ms0606 on the drug resistance of *Mycobacterium smegmatis*. **[Methods]** Firstly, the growth curve was measured to detect the regulatory role of Ms0606 in mycobacterial resistance; electrophoretic mobility shift assay and DNase I footprinting assay were used to identify the conservative sequence recognized by the transcription factor Ms0606, and then explore its potential target genes. Secondly, reverse transcription-qPCR and β-galactosidase activity experiments were used to detect the regulatory effect of Ms0606 on the target gene *Ms0608*. The potential roles of Ms0606 in the regulation of drug resistance are further discussed. **[Results]** Overexpressing *Ms0606* made mycobacteria more sensitive than the wild-type strain in response to isoniazid, whereas disrupting *Ms0606* is resistant to isoniazid; Ms0606 could recognize a conserved 22 bp palindromic motif within the upstream region of its self-operon, using the palindrome sequence to search *M. smegmatis* genome, it is found that Ms0606 may regulate 5 potential target genes; Reverse transcription-qPCR and β-galactosidase activity experiments revealed Ms0606 acts as a repressor and negatively regulates *Ms0608* expression, which may affect mycobacterial antibiotic resistance. **[Conclusion]** I have identified a new TetR family transcriptional regulator of *Mycobacterium smegmatis* encoded by Ms0606, which regulates mycobacterial sensitivity to isoniazid, and further characterized its target genes and its regulatory mechanism for mycobacterial resistance.

Keywords: transcription factor, mycobacteria, isoniazid, antibiotic resistance

Isoniazid (INH) is one of the major first-line drugs for treating a serious infectious disease-tuberculosis (TB), caused by *Mycobacterium tuberculosis*[1]. However, while anti-TB drugs have effectively inhibited the spread of TB, the problem of bacterial resistance is becoming more and more serious[2].

The mechanism of anti-TB drugs in *Mycobacterium tuberculosis* is very complex. Most of the mechanisms are the mutations in drug target genes, in addition to the barrier function of cell wall, the deactivation of antibiotics, changes in drug targets and the increase of drug efflux have been reported[3–4]. However, the regulatory mechanisms of drug resistance, particularly the regulators that directly mediate the drug resistance are not well

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understood in *Mycobacterium tuberculosis*.

Both *Mycobacterium smegmatis* and pathogenic *Mycobacterium tuberculosis* belong to the genus *Mycobacterium*, and have many similarities in physiology and genetics. However, *M. smegmatis* is a non-pathogenic strain with no harm to humans or animals and grows relatively quickly[5–6]. Therefore, these characteristics make *M. smegmatis* an important model strain for studying the drug resistance and transcriptional regulation mechanism of *M. tuberculosis*. Recently, the genome sequence of *M. smegmatis* has been completed, and nearly 500 regulators exist in the *M. smegmatis* genome, including a large group of TetR family regulators[6]. TetR/AcrR family transcription regulator contains the N-terminal DNA binding motif with a helix-turn-helix structure, and C-terminal has a ligand recognition domain, which often act as inhibitors in bacteria and paleontology[7]. It has been reported to mediate drug resistance by regulating some drug transport and resistance-related genes[8]. Therefore transcriptional regulation plays a vital role in the response of bacteria to environmental stress[9].

For example, the TetR in *Escherichia coli* negatively regulate the expression of genes related to tetracycline efflux pump and acts as a repressor[10]. The C-terminus of this family protein is a ligand-binding domain that can bind to tetracycline and transport the tetracycline to the outside of the cell, alleviating the inhibitory effect of TetR on the transport pump[11]. In *M. smegmatis*, LfrR is a transcriptional inhibitor of the TetR-like family, which controls the expression of the drug efflux pump LfrA negatively, thus reducing the efflux of drugs, and caused the strain to be sensitive to various drugs such as ciprofloxacin, norfloxacin and ethidium bromide[12]. Regulating the expression of drug-resistant genes may be a general characteristic of transcriptional repressors. In addition, TetR family transcriptional regulators usually regulate their own expression. Such as Ms6564 in *Mycobacterium smegmatis* regulates the expression of the regulator by binding with its own promoter, evidence show that Ms6564 might serve as a extensive regulator and may be involve in a global regulation of gene expression, including DNA damage repair and cell cycle[13]. However, what I know about transcription factors that respond to the antituberculosis drugs is still limited within the range of *M. smegmatis* and related species of mycobacterium.

In this research, I characterized a new TetR family regulator in *M. smegmatis*, which is encoded by *Ms0606*. The results showed that *Ms0606* specifically bound to its self-promoter by identifying a 22 bp palindromic sequence motif separated by six nucleotides, and its overexpression significantly enhanced bacterial INH sensitivity, whereas its deletion resulted in INH resistance. Thus, I have identified a transcriptional repressor that regulates INH sensitivity in *M. smegmatis.*

1 Materials and methods

1.1 Strains, enzymes, reagents, plasmids

The *Escherichia coli* BL21 strains and vector (pET-28a) needed for protein expression were purchased from Novagen (Germany). *E. coli* XR strains, pBT, pTRG vectors were obtained from Stratagene (USA). Restriction enzymes, T4 DNA ligase, and reagent required for PCR reaction were bought from TaKaRa Biotech (Dalian), all antibiotics chemical reagents were bought from Sigma company. PCR primers I used were obtained from Tsingke and Invitrogen (China).

1.2 Gene cloning, expression and purification of Ms0606 protein

The related genes required in this study were amplified from the *M. smegmatis* mc^2155 genome. Then the resulting products were treated with the corresponding restriction endonucleases, and purified with PCR DNA purification kit (BioFlux). The *Ms0606* gene was cloned into the pET-28a (cm1)

vector and transformed into *E. coli* BL21 strain, the correct sequencing strain was selected for expression, and the activated bacterial cells were grown in 1 L of Kan-containing Luria-Bertani (LB) culture until the OD_{600} was about 0.8–1.0 at 37 °C. Then 0.5 mmol/L isopropy-β-D-thiogalactoside (IPTG) was added to induce protein expression at 30 °C for 4 h. Later the proteins were purified by Ni^{2+} column affinity chromatography. Firstly, the cells were centrifuged at 8000 r/min for 2 min and resuspended in binding buffer (10 mmol/L imidazole; 100 mmol/L NaCl; 20 mmol/L Tris-HCl, pH 8.0), then the supernatant after sonication was passed through Ni^{2+} (50 mmol/L $NiSO_4$) treated His-gel beads. The non-target protein was washed by 10–15 mL buffer (100 mmol/L NaCl; 20 mmol/L Tris-HCl, pH 8.0; 40 mmol/L imidazole). Finally, the Ms0606 protein is eluted with 15–20 mL of elution buffer (100 mmol/L NaCl; 20 mmol/L Tris-HCl, pH 8.0; 250 mmol/L imidazole), then the protein is saved at 4 °C and SDS-PAGE gel was used to detect protein. The protein with higher purity and concentration was selected for dialysis and transferred to 100 μL aliquots per tube, the eluate was marked and stored at –80 °C for later use.

1.3 Bacterial one-hybrid experiment

The coding sequence of Ms0606 was cloned into the pTRG vector, and the 500 bp upstream promoter region of Ms0606 (Ms0606p) and the control gene Ms0612 (Ms0612p) were cloned into the *HIS3-aadA* reporter gene upstream of the one-hybrid reporter vector pBXcmT[14]. Co-transformants were selected on a screening medium which contained 15 μg/mL tetracycline, 16 μg/mL streptomycin, 30 μg/mL kanamycin, 34 μg/mL chloramphenicol, and 20 mmol/L 3-amino-1,2,4-triazole. Bacterial one-hybrid experiment was conducted as previously described[14].

1.4 Electrophoretic mobility shift assays (EMSA)

Long fragments over 500 bp were amplified by PCR from *M. smegmatis* mc^2155 genomic DNA. Subsequently, the obtained products were purified by DNA purification kit and 5′-terminus labeled with the fluorescein isothiocyanate (FITC). The short segments below 50 bp were directly annealed *in vitro*. The EMSA assays were operated as reported procedure[13] with several changes. The 1 μL labeled DNA fragment were co-incubated with concentration gradient of the protein in a total volume of 20 μL EMSA buffer (50 mmol/L Tris-HCl pH 7.5, 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT), then the mixtures were reacted at 4 °C for 30 min and conducted 5% native Polyacrylamide Gel Electrophoresis (5 mL 40% acrylamide, 31 mL ddH_2O, 300 μL APS and 50 μL TEMED) at 150 V for 1–2 h. Finally it was put into the Typhoon Scanner (GE Healthcare) fluorescence to scan, then saved the results after analyzing with image processing software.

1.5 DNase I footprinting assay

As mentioned above in reference [15]. The promoter region of the *Ms0606* gene was amplified with primers labeled with FITC and purified with BioFlux DNA Purification kit. Under the same conditions as the above EMSAs, just add 1–2 μL of DNase I to the system and expand the reaction to 150 μL, then each mixture was co-incubated at 37 °C for 1–2 min. The final sample was performed by an Applied Biosystems 3730XL DNA instrument of Wuhan Qingke Company, and the electropherograms of sequencing analysis was analyzed and drawn with GENEMAPPER software (version 4.0).

1.6 Southern blotting

The wild-type *M. smegmatis* mc^2155 and *Ms0606*-deleted strains were collected in the mid-log phase, and the total DNA was extracted using the TIANGEN bacterial genomic DNA extraction kit (spin column type), then the qualified total DNA was digested by restriction enzyme *Sph* I, 0.8% agarose gel was used to concentrate and separate the total DNA. After the electrophoresis was completed, it was denatured with 0.4 mol/L NaOH. Finally, after the membrane was transferred,

the reaction was carried out according to the instructions of the Roche digoxin labeling southern hybridization kit. The specific experimental method was referred to the published literature[16].

1.7 Quantitative PCRs analysis

The real-time PCR analysis follow the procedure described above[17]. mRNA was extracted from *M. smegmatis* wild-type (Ms and Ms/pMV261) and *Ms0606* recombinant strains (Ms/△Ms0606 and Ms/pMV261-*Ms0606*). Briefly, the strains were cultured to the OD_{600} is about 0.8, and the RNA is extracted by bacterial RNA kit (see the kit for details). 0.8% agarose gel is used to detect the purity and integrity of RNA samples. The cDNA obtained after reverse transcription is subjected to RT-qPCR reaction: 10 μL 2×SYBR Green Master Mix Rergent, 3 μL cDNA, 1 μL gene-specific primers, 6 μL deionized water. The reactions were reacted in a CFX96 instrument (Bio-Rad, USA) using the following protocol: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The Bio-Rad CFX Manager v.2.1 software was used to analyze the data. Expression levels of different genes were normalized by *sigA* and RT-qPCR datas were calculated using the $2^{-\Delta\Delta Ct}$ method[18].

1.8 β-galactosidase activity analysis

The β-galactosidase activity experiment was carried out in *M. smegmatis*, a series of pMV261-vectors were constructed with *LacZ* as the reporter gene, Ms0608p and Ms0612p were digested and ligated into pMV261-vector[19]. Recombinant plasmids pMV261-Ms0608p-lacZ and pMV261-Ms0612p-lacZ were transformed into the wild-type *M. smegmatis* and *Ms0606*-knockout strains. All report strains were grown in 7H9 liquid medium at 37 °C for two days until OD_{600} to 1.0, then cell suspension was collected and β-galactosidase activity was measured as before[20].

1.9 Determination of mycobacterial growth curves and the MIC of drugs

When the *Ms0606* recombinant strains grew to OD_{600} between 1.5 and 1.8, the fresh bacteria were transferred to 100 mL 7H9 medium adding the 5 μg/mL INH. Then the cultures (OD_{600}=0.15) were shaken at 160 r/min for 3–4 d at 37 °C for further growth, and these samples were harvested and measured at the same time for OD_{600}.

Minimal inhibitory concentration (MIC) determination using Kirby-Bauer test method[21] with several changes. The strain to be tested was coated on the 7H10 plate, and then 20 μL of 0.64, 1.28, 2.56, 5.12 μg/mL, 10.24 μg/mL INH were added to the filter paper with a diameter of 1 cm. After the filter paper was dried, the filter paper with different drug concentration was placed on the coated plate with bacteria, allowing the drug could spread on the plate. The plate was incubated at 37 °C for 48 h, and the inhibitory zone around the filter paper was observed. The size of the inhibition zone reflects the sensitivity of the tested bacteria to the INH, and is negatively correlated with the minimum inhibitory concentration (MIC) of the INH, that is, the inhibition zone is large and the MIC of INH for *M. smegmatis* strain is small.

2 Results

2.1 Ms0606 potentially modulates the sensitivity of *Mycobacterium smegmatis* to INH

In order to recognize the potential transcription factors regulating the drug resistance in *Mycobacterium smegmatis*, I selected the library of *M. smegmatis* mc^2155 (Accession number CP000480) under the strong promoter *hsp60*. I cloned approximately-500 transcription factors of *M. smegmatis* into the overexpressing plasmid pMV261 and the recombinant strains were detected on 7H10 solid medium with 2.5 μg/mL INH. A hypothetical transcription factor encoded by *Ms0606* was isolated, potentially leading to the INH sensitivity of *M. smegmatis*. As shown in Figure 1, when the recombinant strains were 10×fold diluted to two different concentrations and spotted on 7H10 plates

without (left panel) or with 2.5 μg/mL INH (right panel), compared with the wild-type strain with blank vector pMV261(Msm/pMV261), the *Ms0606*-over expressed strain transformed with pMV261-*Ms0606* (Msm/pMV261-*Ms0606*) was more sensitive to INH (Figure 1, right panel), the two recombinant mycobacterial strains grew equally on the plate without the drug (Figure 1, left panel).

Furthermore, the minimal inhibitory concentrations (MICs) of *M. smegmatis* wild-type and *Ms0606*-overexpressed strains on INH were determined in the existence of INH with different dilution gradients. As shown in Figure 2 and Table 1, in the presence of INH in the culture medium, the inhibition zone diameter of INH to *Ms0606*-overexpressed strain was 25 mm, and the diameter of wild-type strain was 20 mm. This observation suggests that the *Ms0606* could increase the INH sensitivity of *M. smegmatis*. A sequence analysis showed that the *Ms0606* gene encodes a 209-residue protein, which contains a typical TetR N-terminal helix-turn-helix structure in the AcrR region (Figure 3). Therefore, Ms0606 belongs to the TetR/AcrR family of transcription factors[22].

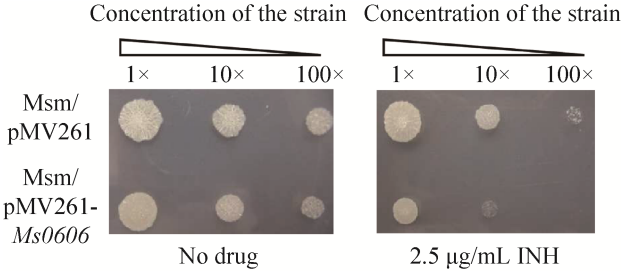

Figure 1. The effect of Ms0606 on the INH sensitivity of *M. smegmatis* and its domain structure.

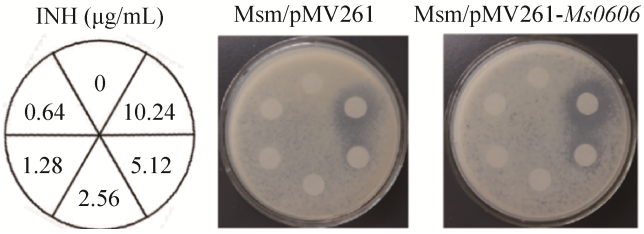

Figure 2. The MIC determination in Msm/pMV261 and Msm/pMV261-*Ms0606* strains.

Table 1. Inhibition zone diameter of INH to *M. smegmatis* (Msm)

	Msm/pMV261	Msm/pMV261-*Ms0606*
Inhibition zone diameter/mm	20	25

Inhibition zone of INH to Msm/pMV261 and Msm/pMV261-*Ms0606* was measured by Kirby-Bauer test method.

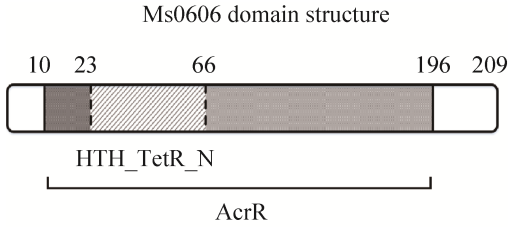

Figure 3. The domain structure of Ms0606 protein.

To confirm the regulating effect of Ms0606 on the growth of *M. smegmatis* under the action of INH, I measured the growth curves of the wild-type (Msm/WT, Msm/pMV261), *Ms0606*-knockout strain (Msm/△Ms0606) and *Ms0606*-overexpressed strain (Msm/pMV261-*Ms0606*). As shown in Figure 4-A, *Ms0606*-overexpressed strain grew dramatically slowly than the wild-type strain in 7H9 liquid medium with 5 μg/mL INH (Figure 4-B), * $P \leqslant 0.05$. Besides, no significant difference was found in these two strains (Figure 4-A). But in contrast, the *Ms0606*-deleted *M. smegmatis* strain grew only a modest better than the wild-type strain under the same conditions (Figure 4-D), and these two strains were similar in the absence of INH (Figure 4-C).

Therefore, these results indicated that Ms0606 may be involved in the regulation of INH sensitivity in *M. smegmatis*.

2.2 Ms0606 binds its own promoter

The binding of Ms0606 to its own promoter was also detected because most of the TetR family transcriptional factors presented a self-regulation mechanism in mycobacteria. To test this idea, firstly, a bacterial one-hybrid system was used[14] to detect DNA-protein interactions through transcriptional activation effects of the reporter genes *HIS3* and *aadA* (Figure 5-A). When co-transformants were spotted on the plates with or without the selective drugs 3-AT and

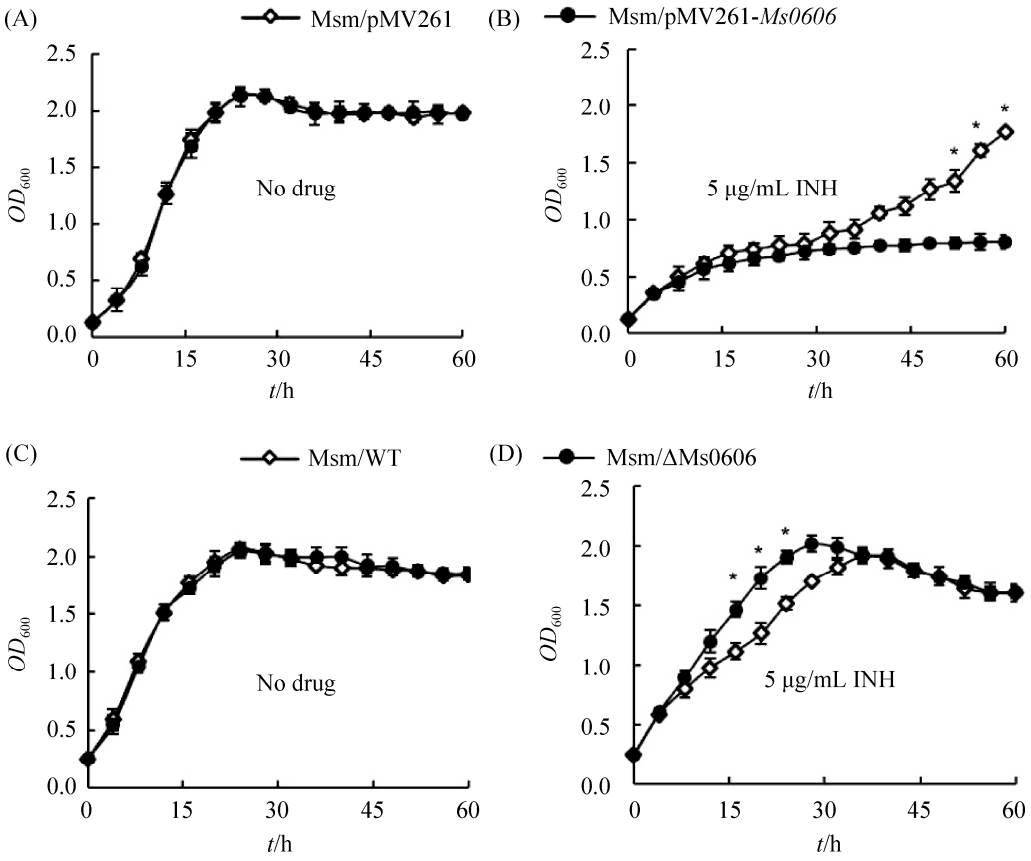

Figure 4. Growth analysis of evaluating the effect of Ms0606 on INH sensitivity in *M. smegmatis*. Growth curves of wild-type (Msm/pMV261) and *Ms0606*-overexpressing (Msm/pMV261-*Ms0606*) strains in the absence (A) and presence of (B) 5 μg/mL INH; Growth curves of the wild-type (Msm/WT) and *Ms0606*-deleted (Msm/ΔMs0606) strains in the absence (C) and presence of (D) 5 μg/mL INH. *: $P \leqslant 0.05$.

Str^r (see Materials and Methods), if the regulatory factor cloned into the pTRG vector can activate the promoter cloned into the pBXcmT vector, the reporter genes would be induced and therefore the co-transformants grow well on the screening medium (+3-AT, $+Str^r$). As shown in Figure 5-B, both the positive control (CK+) and pTRG-Ms0606/pBX-Ms0606p grew very well in the selective plate (Figure 5-B). On the contrary, no colony growth was observed for the negative control (CK–) and the self-activation control including either Ms0606 or the promoter alone, indicating that Ms0606 can bind with its own promoter, Ms0606p.

Secondly, electrophoretic mobility shift assays (EMSAs) were used to further confirm the results *in vitro*. As shown in Figure 5-C, when 0.3 nmol/L promoter DNA (Ms0606p) and a control DNA substrate (Ms0612p) were incubated with Ms0606 protein, stable protein-DNA complex bands were observed as the protein concentration (1–8 μmol/L) in the reaction system increased (Figure 5-C, lanes 2–5), but not negative control Ms0612p (Figure 5-C, lanes 7–10). Additionally, a competition assay was performed to determine the specificity of Ms0606 binding with Ms0606p. As shown in Figure 5-D, when the concentration of unlabeled Ms0606p was gradually increased in the reaction system, the binding of FITC-labeled Ms0606p to Ms0606 protein could be competitively inhibited (Figure 5-D, lanes 7–9), but not unlabeled Ms0612p under the same conditions (Figure 5-D, lanes 10–12).

The above results indicated that Ms0606 can recognize its self-promoter specifically.

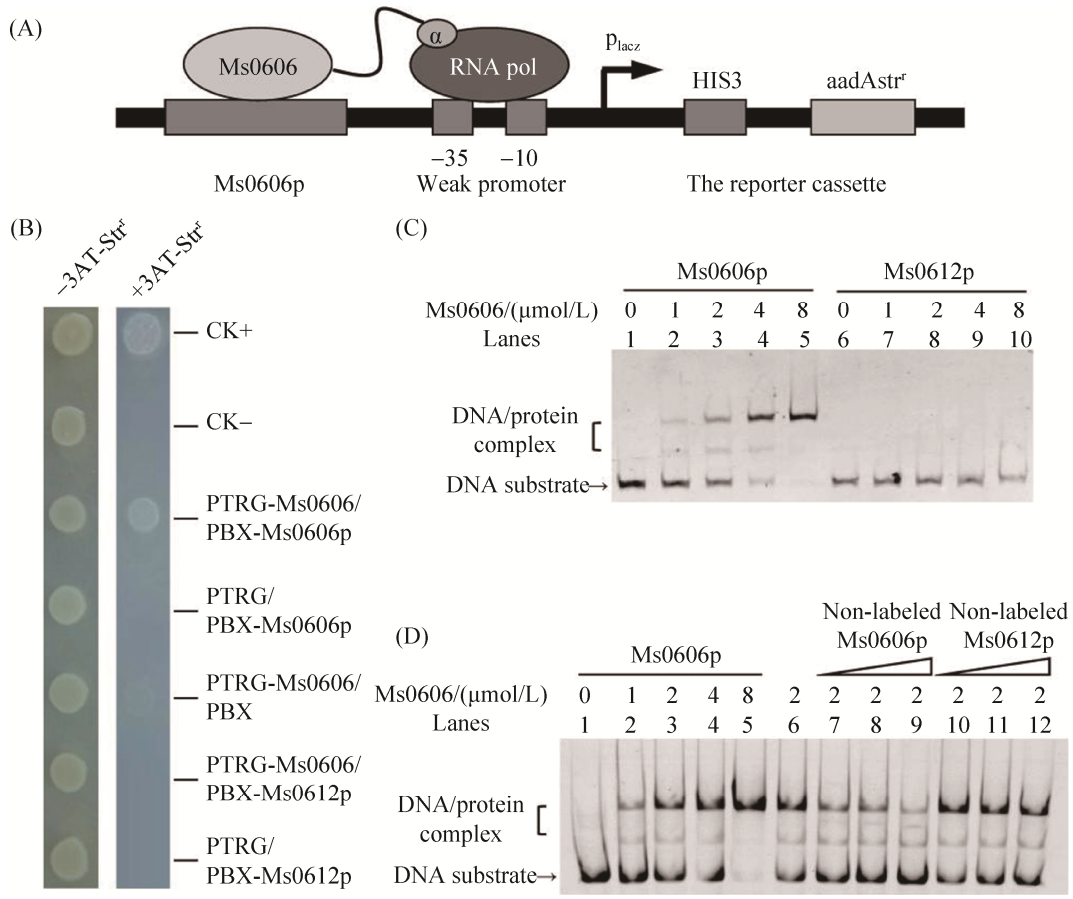

Figure 5. Ms0606 binds to its own promoter specifically. A: The putative promoter region of Ms0606 was cloned into the *HIS3-aadA* reporter genes upstream of the bacterial one-hybrid reporter vector pBXcmT; B: Bacterial one-hybrid assays; C: EMSA assays; D: EMSA assays for the specific binding of Ms0606 to its own promoter.

2.3 Ms0606 identifies a palindromic sequence motif

To further map the DNA binding motif identified by Ms0606, several truncated DNA substrates covering the promoter region of Ms0606 were produced and designated as S1, S2, S3. As shown in Figure 6, an obvious DNA-protein complex was observed only for

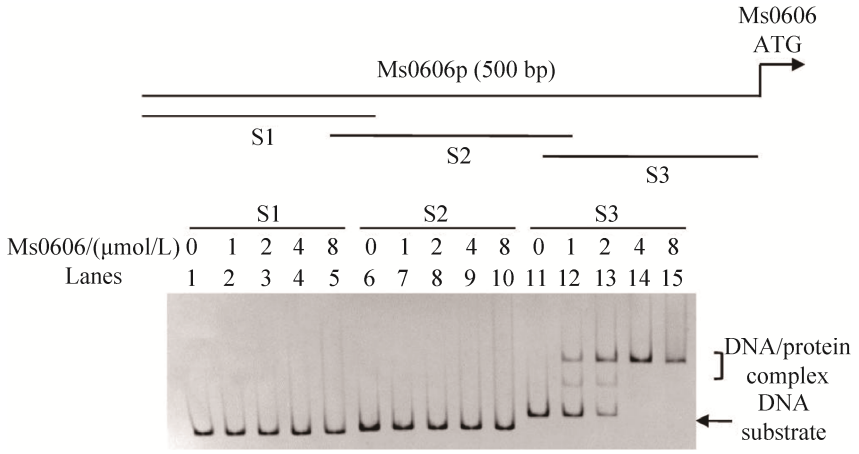

Figure 6. EMSA detected the DNA-binding fragments of Ms0606 in the upstream regions of *Ms0606*.

S3 (lanes 11–15) in EMSA assays, but not fragment S1 or S2 (lanes 1–10), indicating that the potential binding motif for Ms0606 should be in the region S3 fragment.

Then DNase I footprinting assay was used to precisely recognize the binding motif of Ms0606. The FITC-labeled S3 fragment and Ms0606 protein were co-incubated and digested with DNase I. As shown in Figure 7-A, as the Ms0606 protein (0–4 μmol/L) concentration increased, the area around GAAA**CTGAATCT**AACATC **AGATTCAG**GTTG protected by Ms0606 became more and more obvious (Figure 7-B). The region contained two inverted repeats (IRs) and separated from each other by six nucleotides.

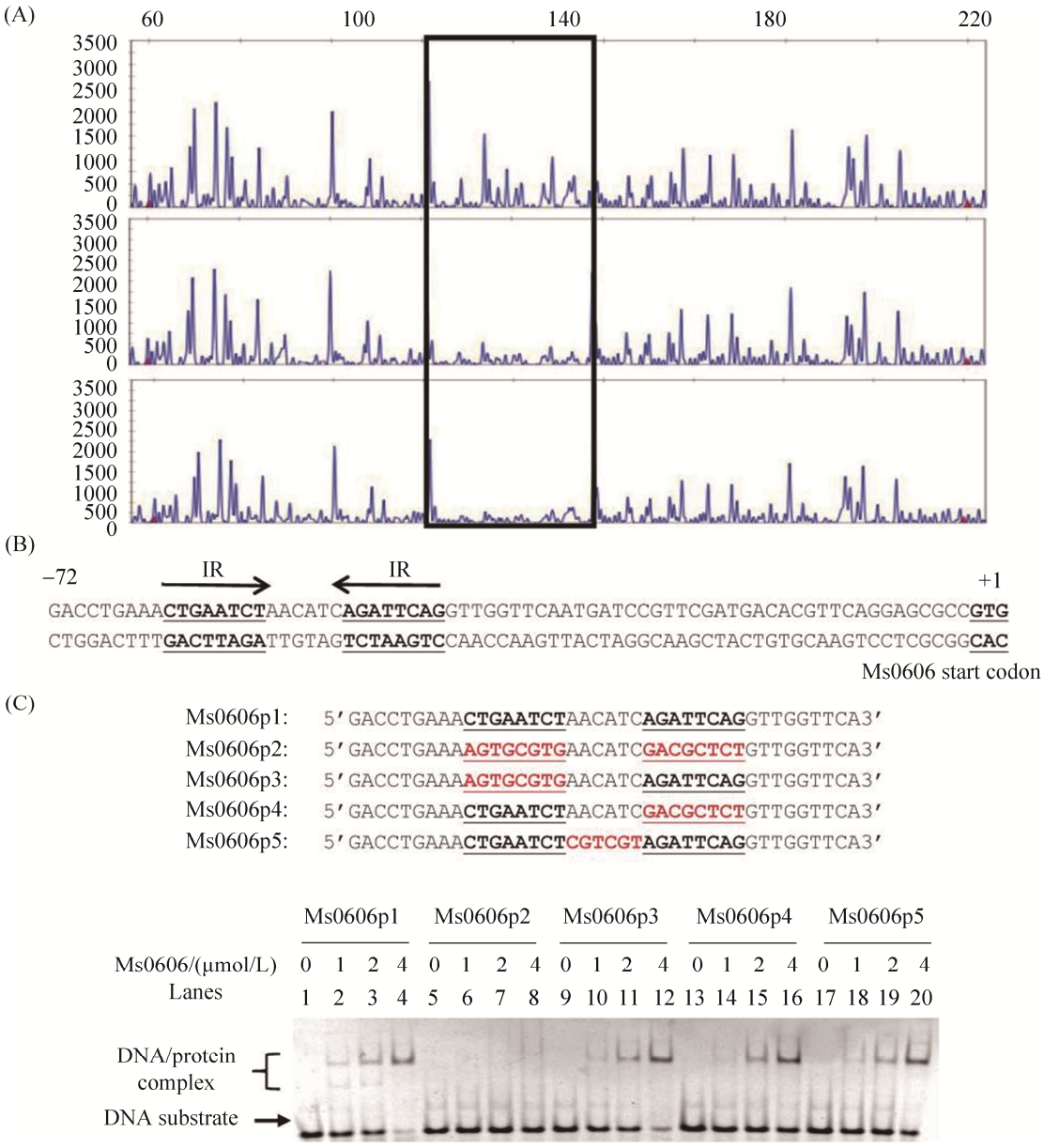

Figure 7. Identification of the DNA motif bound for Ms0606 with Ms0606p. A: DNase I footprinting experiments; B: sequence structure of the protected DNA region; C: EMSA for the DNA-binding activity of Ms0606 to the Ms0606p1, Ms0606p2, Ms0606p3, Ms0606p4 and Ms0606p5.

In order to further confirm the specificity of the motif identified by Ms0606. In the EMSA assays, I synthesized a series of about 40 bp fragments containing binding sites or motif mutations oligonucleotides (Figure 7-C). As shown in Figure 7-C, Ms0606 can't combine Ms0606p2 in which the two inverted repeats were mutated by random sequences (Figure 7-C, lanes 6–8). In contrast, neither a portion of the IRs nor a mutation in the interval sequence has eliminated their interaction (Figure 7-C, 10–12, 14–16, 18–20), and only one binding band was observed in mutants, suggesting that Ms0606 may be combined with the promoter in two steps.

In summary, my results indicate that Ms0606 can recognize a conserved 22 bp palindromic sequence motifs (**CTGAATCT**NNNNNN**AGATTCAG**).

2.4 Several genes promoter region may contain the binding motif for Ms0606

Through the identification of the binding motif of Ms0606, I can find the potential target genes regulated by the protein Ms0606. The entire 500 bp upstream promoter region of the open reading frame was searched in the *M. smegmatis* genome for the binding motif of Ms0606 with partial base mismatch, five promoters (Ms1969, Ms2075, Ms0608, Ms0801 and Ms6834) was searched to contain the potential Ms0606 binding site, in addition to the self-promoter of Ms0606, indicating that these genes may be regulated by Ms0606. This leads us to assume that Ms0606 may be a regulator of these potential target genes. To test this assumption, the promoter DNA of Ms1969, Ms2075, Ms0608, Ms0801 and Ms6834 were synthesized and detected by EMSA assays. As shown in Figure 8, When putative promoters DNA and Ms0606 protein were co-incubated, with the increase of protein concentration (0–4 μmol/L), the promoter of Ms1969, Ms2075 and Ms0608 form a stable complex with Ms0606 (Figure 8, lanes 1–12). But in the promoter of Ms0801 and Ms6834, only faint shifted band was observed (Figure 8, lanes 13–20), suggesting that Ms0606 may have different ability to bind promoters.

These findings suggest that the five genes may be the potential target gene of Ms0606 in *M. smegmatis*.

2.5 Ms0606 is a transcriptional repressor that negatively regulates the expression of the *Ms0608*

To further study the regulatory function of Ms0606, I used homologous recombination exchange to construct the *Ms0606* knockout strain in the *M. smegmatis* strain (Figure 9-A). As shown in Figure 9-A, the 1 kb upstream and downstream of the *Ms0606* gene was cloned into a pMind-derived[23] suicide plasmid by homologous recombination, then the constructed knockout vector was transferred into *M. smegmatis* wild-type strain to obtain *Ms0606*-deleted strain (Figure 9-B). Further Southern blotting[24] was used to verify whether *Ms0606* was knocked out. As shown in Figure 9-C, a schematic diagram of genomic DNA of wild-type and *Ms0606* knock-out strain after digestion with *Sph* I restriction enzyme, and then hybridized with digoxin-labeled probe. As a result, 1.3 kb were detected in wild-type strain,

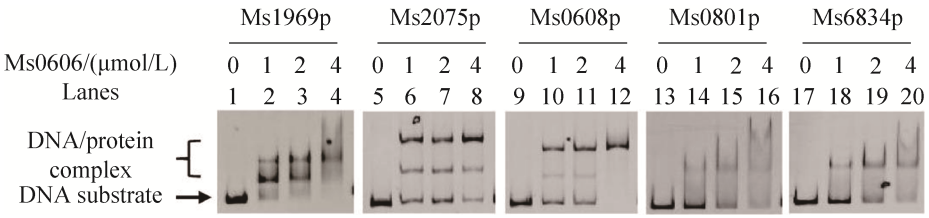

Figure 8. EMSA detected the binding of Ms0606 with the promoters of Ms1969, Ms2075, Ms0608, Ms0801 and Ms6834.

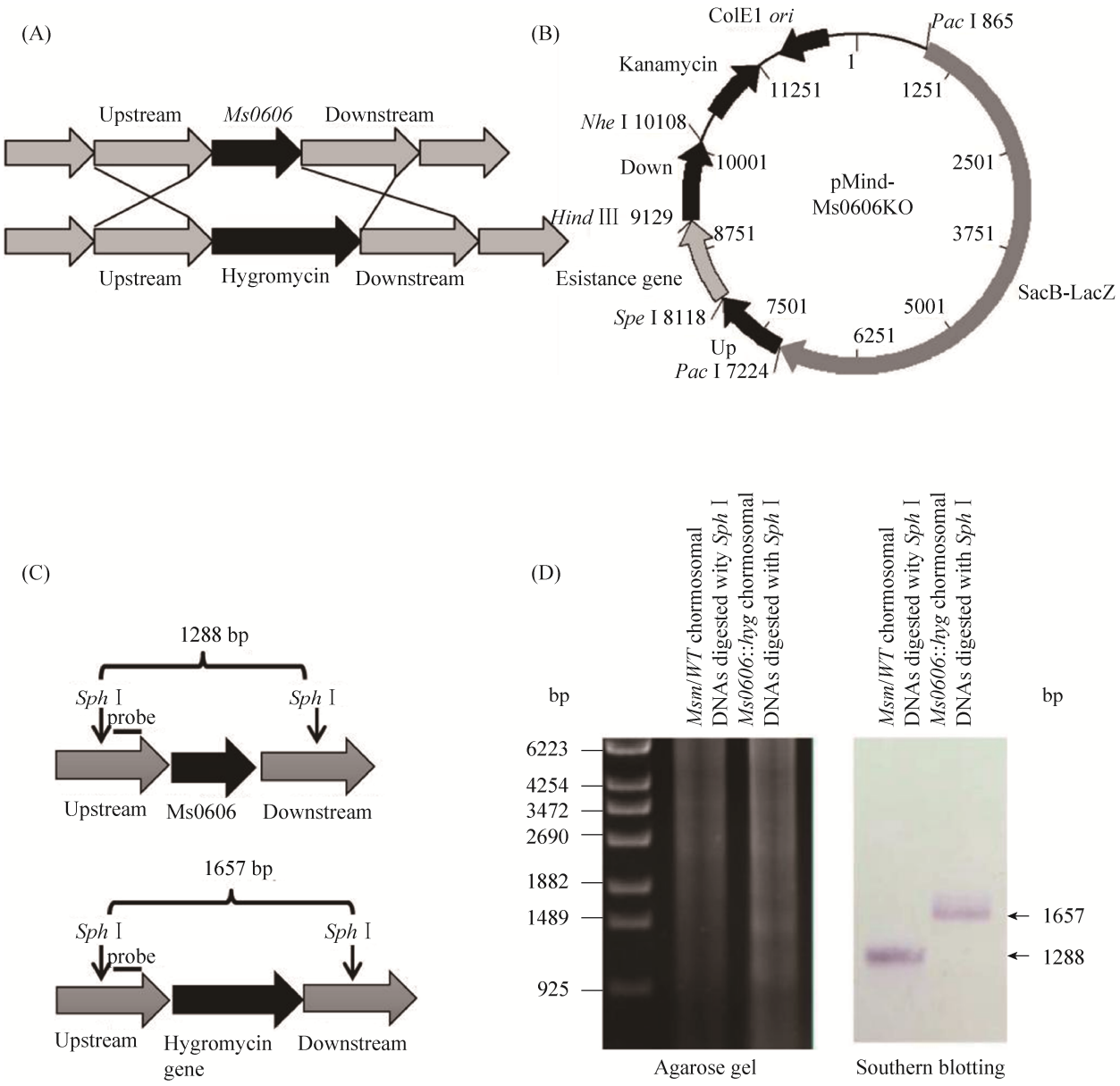

Figure 9. Construction of the *Ms0606* knockout strain in *M. smegmatis* and Southern blot analysis. A: schematic diagram of the recombination strategy; B: a map of the recombinant vector pMindMs0606KO; C: schematic diagram of the DNA fragments of the wild-type and *Ms0606*-deleted strain digested with the restriction enzyme *Sph* I; D: Southern blotting assays.

a band of 1.7 kb size was detected in the *Ms0606*-deleted strain, indicating that *Ms0606* gene was successfully knocked out in the mutant strain (Figure 9-D).

Subsequently, I compared expression levels of the adjacent converse gene, *Ms0608*, in wild type strain and *Ms0606* knock-out strain using RT-qPCR. As shown in Figure 10-A, compared with the wild-type strain, the expression level of *Ms0608* was up-regulated more than twice (P<0.05) in the *Ms0606*-knockout strain. Similarly, I detected the expression level of *Ms0608* in *Ms0606* overexpressing

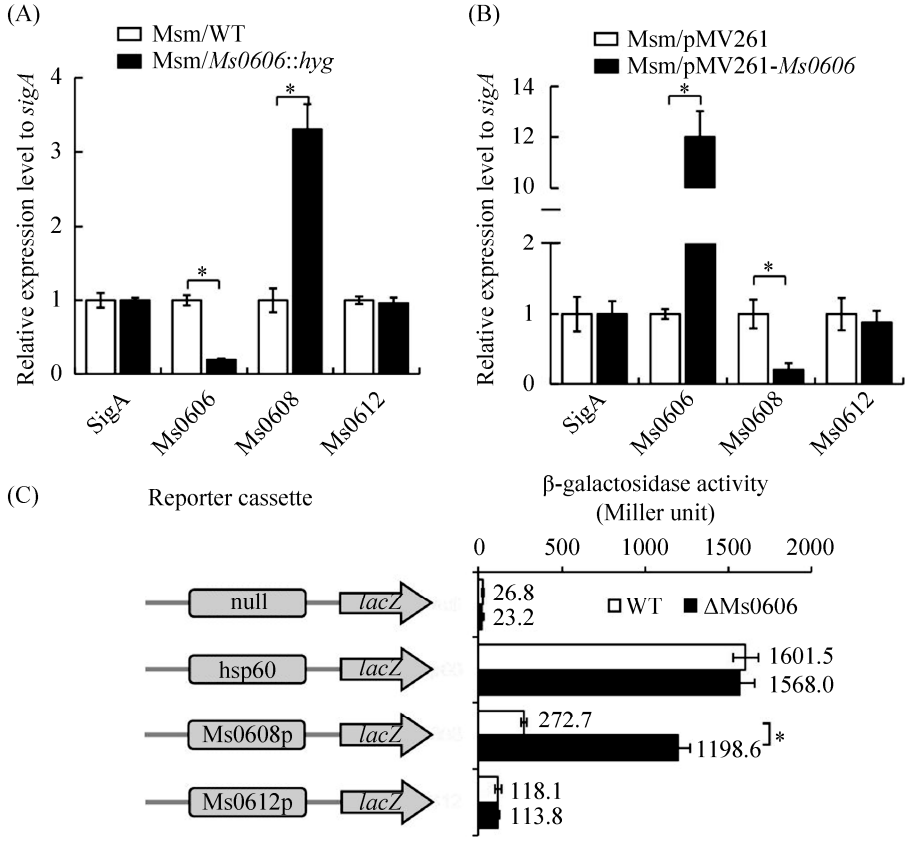

Figure 10. The effect of the Ms0606 regulator on the expression of Ms0608. A: real-time quantitative PCR detection in the Ms0606-knockout strain; B: real-time quantitative PCR detection in the overexpressed strain; C: β-Galactosidase activity assay. *: $P \leqslant 0.05$.

strain containing a pMV261-derived recombinant plasmid. Compared with the control strain, the expression of *Ms0608* in the overexpressed strain was reduced to less than 0.5 times (*P<0.05) (Figure 10-B), which coincided the previous result. Therefore, RT-qPCR experiments proved that Ms0606 acts as a transcriptional repressor, which negatively regulates the expression of *Ms0608* gene.

Further β-galactosidase activity assays confirm this conclusion, a series of promoter-*lacZ* reporter strains were constructed in *M. smegmatis*. As shown in Figure 10-B, compared with the CK-plasmid containing the non-promoter *lacZ* plasmid, the *hsp60* promoter strongly up-regulated the expression of *lacZ* (CK+), indicating that the reporter system is working properly. As expected, compared with the wild type strain, the expression of *lacZ* under the Ms0608 promoter induced by the Ms0606 was increased 5-fold in the *Ms0606*-deleted *M. smegmatis* strain (*P<0.05). This finding is consistent with the results of RT-qPCR described above.

Based on the above results, Ms0606 acts as a repressor and negatively regulates *Ms0608* gene expression in *M. smegmatis*.

3 Discussion

The molecular network of *M. tuberculosis* in response to anti-tuberculosis drugs and its inherent regulation mechanism of resistance to isoniazid are not clear[6]. In this study, I reported a TetR family regulator Ms0606 in *M. smegmatis*, overexpressing *Ms0606* enhances the sensitivity of mycobacteria to INH, while destroying *Ms0606* makes the mycobacteria slightly resistant to INH, which possible "Genetic compensation response (GCR)"[25], that is, after *Ms0606* gene is

mutated and completely loses its function, the bacteria will adopt a corresponding mechanism to increase the expression of other genes to replace the function of *Ms0606*. Therefore, I've uncovered a new transcriptional factor that affects the INH sensitivity of *M. smegmatis*.

The TetR/AcrR regulatory family is widely distributed in many bacteria and usually functions as a suppressor. It plays a regulatory role in bacterial resistance, pathogenicity, antibiotic synthesis and osmotic stress[7]. The acrR operon contains *acrR*, *acrA* and *acrB*, three genes in *E. coli*, the latter two are multidrug-resistant efflux pumps[26–27]. In contrast, Ms0606 contain a typical AcrR domain, but its target is unknown and will be elucidated. In this study, my EMSA data suggest that Ms0606 can bind to the promoter regions of some genes (Ms1969, Ms2075, Ms0608, Ms0801 and Ms6834) and act as a repressor, possibly regulating the expression of these genes.

To further explain the resistance of bacteria, I try to confirm the function of protein encoded by *Ms0608* in *M. smegmatis*. Bioinformatic analysis indicates that Ms0608 belongs to vicinal-oxygen-chelate (VOC) superfamily, which contains motifs that provide a metal binding site to promote metal ions to participate in the catalysis of enzymes, including the glyoxalase I, type I extradiol dioxygenases and a group of antibiotic resistance proteins[28]. Although the proteins of this superfamily are functionally different, their structures are similar. Antimicrobial resistance proteins use multiple mechanisms to block the function of antibiotics. For example, bleomycin resistant protein (BLMA) inhibits its activity by directly binding to bleomycin[29], glyoxalase I and BLMA exhibit domain exchange between subunits. Whereas, three fosfomycin resistant proteins inactivated fosfomycin by modifying the fosfomycin molecule[30]. Moreover, I tried to identify orthologs of Ms0608 based on a BLAST search, as shown in Figure 11, Ms0608 protein

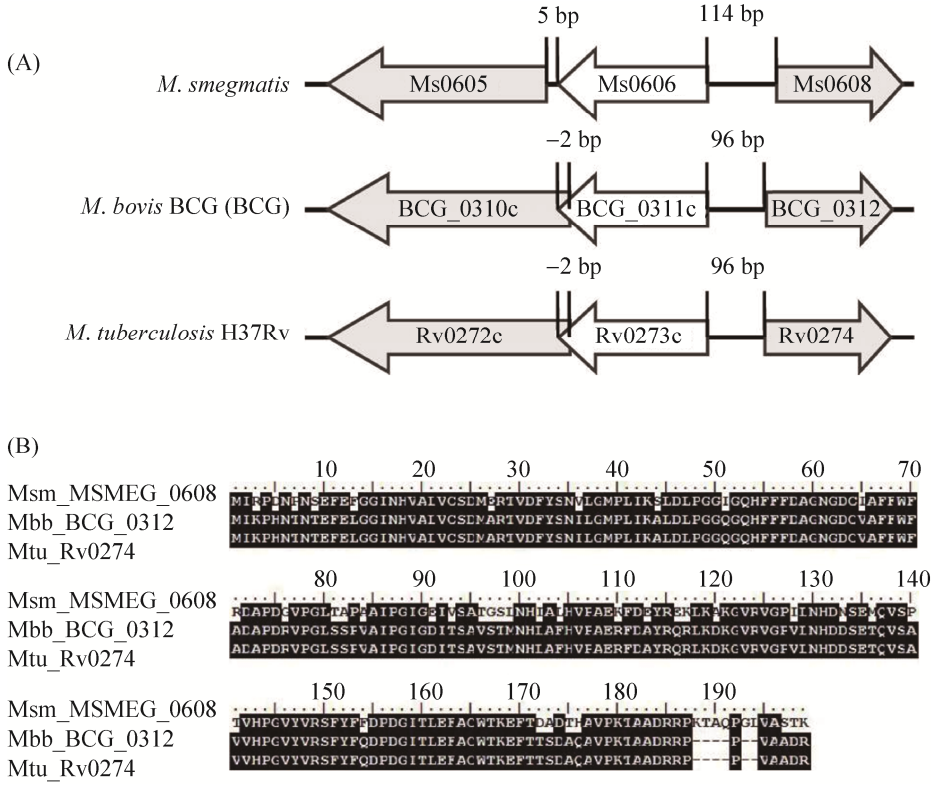

Figure 11. Ms0608 are conserved in mycobacterial species. A: the arrangement of Ms0608 and its adjacent genes in *Mycobacterium* genome; B: the Ms0608 protein orthologs in *M. tuberculosis*, *M. bovis BCG* (100% amino acid identity) and *M. smegmatis* (79% amino acid identity).

was identified 79% amino acid sequence similarity with orthologs in *M. tuberculosis* H37Rv and *M. bovis* BCG (100% amino acids are identical throughout the length of the protein). It has been reported Rv0274 may encode a novel detoxification protein in *M. tuberculosis*[31], indicating Ms0608 protein may has a similar protective effect against toxins and antibiotics. I have proved that Ms0606 negatively regulates expression of Ms0608, although the details were not very clear, it is reasonable that Ms0606 may affect bacteria sensitivity to drugs by regulating the expression of Ms0608. In addition to Ms0608, while others (Ms1969, Ms2075, Ms0801 and Ms6834) may also affect drug resistance, which may play a role in multiple stress adaptations in mycobacteria. Therefore, the underlying mechanism of regulation of antibiotic resistance formation remains to be further studied in the future.

4 Conclusion

In summary, a TetR/AcrR family of transcription factors, Ms0606, was turned out to be a transcription repressor and negatively regulate INH resistance in *M. smegmatis*. Ms0606 specifically identified a 22 bp palindromic sequence motif separated by a 6 bp spacer. Notably, I found that Ms0606 regulates the expression of the Ms0608 gene. Apparently, my findings provide important clues for further studying a link of a transcriptional regulator Ms0606 and mechanism of antibiotic in mycobacteria.

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耻垢分枝杆菌中一个 TetR 家族转录因子负调控异烟肼的敏感性

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摘要：【目的】研究 TetR 家族转录因子 Ms0606 对耻垢分枝杆菌耐药性的调控作用。【方法】首先，通过测定生长曲线检测 Ms0606 在分枝杆菌耐药性中的调控作用；通过凝胶迁移阻滞实验和 DNase I 足迹法鉴定转录因子 Ms0606 识别的保守序列，进而探究其潜在的靶基因；其次，利用逆转录-qPCR 和 β-半乳糖苷酶活性实验检测 Ms0606 对靶基因 *Ms0608* 的调控作用，进一步探讨 Ms0606 调控分枝杆菌耐药性的分子机制。【结果】与野生型菌株相比，*Ms0606* 超表达菌株对异烟肼表现为敏感，*Ms0606* 敲除菌株对异烟肼表现为抗性；Ms0606 可以识别其自身操纵子上游区域内保守的 22 bp 回文序列，利用回文序列搜索耻垢分枝杆菌的基因组，发现 *Ms0606* 可能调控 5 个潜在靶基因；逆转录-qPCR 和 β-半乳糖苷酶活性实验显示 Ms0606 作为抑制子负调控 *Ms0608* 的表达，这可能会影响分枝杆菌对药物的耐药性。【结论】鉴定了一种新的由 Ms0606 编码的耻垢分枝杆菌的 TetR 家族转录调控因子，该因子可调节分枝杆菌对异烟肼的敏感性，并进一步探讨其对靶基因的调控功能及对分枝杆菌耐药性的调控机制。

关键词：转录因子，分枝杆菌，异烟肼，抗生素耐药性

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