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Research Article 研究报告

Ethambutol and isoniazid influence the growth of *Mycobacterium smegmatis*

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Abstract: [Objective] Isoniazid (INH) and ethambutol (EMB) are the two main first-line drugs for the treatment of tuberculosis (TB), while the underlying synergistic mechanism remains unclear. **[Methods]** The *Ms0606* gene was cloned from *Mycobacterium smegmatis* and its expression was induced by the addition of 0.5 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 h. The interaction between Ms0606 protein and EMB was studied by isothermal titration calorimetry (ITC). The effect of EMB on the DNA binding activity of Ms0606 was examined by electrophoretic mobility shift assay (EMSA). The growth curves of the *Ms0606*-overexpressing strain treated with INH and INH+EMB were established. **[Results]** Ms0606 protein was purified with high purity. EMB can bind with Ms0606 at a ratio of 1:1 and specifically enhance the DNA binding activity of Ms0606. In the presence of a non-lethal dose of EMB, the overexpression of *Ms0606* enhanced the effect of INH on *M*. *smegmatis* MC^2155. **[Conclusion]** The TetR family transcriptional regulator encoded by *Ms0606* plays a role in the synergistic effect of EMB and INH, the further research on which will help us to understand the regulatory mechanism of bacterial resistance.

Keywords: ethambutol; transcription factor; interaction; drug resistance; drug combination

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乙胺丁醇与异烟肼协同对耻垢分枝杆菌生长的影响研究

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ZHU Chen, YANG Min. Ethambutol and isoniazid influence the growth of *Mycobacterium smegmatis*[J]. Acta Microbiologica Sinica, 2023, 63(6): 2430-2439.

摘　要：【目的】异烟肼(isoniazid, INH)和乙胺丁醇(ethambutol, EMB)是治疗结核病(tuberculosis, TB)的 2 种主要一线药物，虽然这 2 种药在临床上的联合使用有效地遏制了结核病的蔓延，但是它们之间潜在的协同作用机制却十分不清楚。【方法】本项目以耻垢分枝杆菌为模式菌株，首先，克隆 *Ms0606* 基因并以异丙基 β-D-1-硫代吡喃半乳糖苷(isopropyl β-D-1-thiogalactopyranoside, IPTG)诱导蛋白质的表达，再采用等温滴定量热法(isothermal titration calorimetry, ITC)证实 Ms0606 蛋白与 EMB 存在相互作用，进一步通过凝胶迁移实验(electrophoretic mobility shift assay, EMSA)证明 EMB 对 Ms0606 的 DNA 结合活性的影响，然后检测基因 *Ms0606* 超表达菌株在 INH 及 INH+EMB 处理下细菌的生长曲线。【结果】最终纯化得到纯度较高的 Ms0606 蛋白，EMB 与 Ms0606 以 1:1 的比例结合，并且 EMB 能特异性地增强 Ms0606 的 DNA 结合活性，在非致死剂量 EMB 存在下，*Ms0606* 过量表达增强了 INH 对 *M. smegmatis* MC^2155 的杀菌能力。【结论】发现由 *Ms0606* 编码的 TetR 家族转录调控因子，在 2 种药物协同作用中发挥作用，进一步研究将有助于我们对细菌抗药性调控机制的了解。

关键词：乙胺丁醇；转录因子；相互作用；耐药性；联合用药

The mechanisms of drug resistance in *Mycobacterium tuberculosis* are very complex, and most mechanisms are mutations in drug target genes, except for the already reported impairment of bacterial cell wall barrier function, antibiotic inactivation, altered drug targets, and increased drug efflux[1-2]. However, it is not very clear about the signaling pathway that first-line anti-tuberculosis (TB) drugs mediate mycobacterial resistance, especially the regulatory mechanism directly mediated by transcription factors.

Transcription factors mediate bacterial drug resistance by regulating some drug transport and drug resistance-related genes, and play very important regulatory roles in bacterial life activitiy[3]. Members of the TetR/AcrR family contain N-terminal DNA-binding domain and C-terminal ligand binding domain, and the C-terminal domain enables the TetR/AcrR family transcription factors to accommodate multiple ligand bindings by identifying different compounds[4]. Therefore, TetR/AcrR family transcription factors, like chemical sensors, play important roles in multiple signaling pathways,

including antibiotic production, changes in osmolarity, and expression of efflux pumps, by combining small detection of changes in the intracellular environment and regulating the expression of target genes[5].

For example, TetR transcription factor regulates the expression of tetracycline efflux pump genes. Tetracycline binds to TetR into cells, causing protein conformational changes and promoting tetracycline efflux, thus protecting bacterial cells from toxic substances[6]. QacR, a multidrug-binding transcriptional repressor in pathogenic bacteria *Staphylococcus aureus*, modulates the transcriptional level of the multidrug transporter gene *qacA*, in response to engaging a set of diverse ligands[7]. FadR in *Sulfolobus acidocaldarius* controls a variety of genes in the pathway of fatty acid biosynthesis and degradation by responding to small molecule ligands long-chain fatty acyl-CoA. FadR dynamically regulates fatty acid metabolism and maintains the normal growth and reproduction of microorganisms[8]. In *M. tuberculosis*, fatty acid metabolism also significantly affects pathogenicity[9]. Furthermore, an ArsR family transcription regulator encoded by *Rv2642* (CdiR) responds to isoniazid (INH). The bindings of INH and Cd(II) both reduce CdiR DNA-binding activity, causing sensitivity to INH. CdiR has a critical role in directing the interplay between Cd(II) metal ions and drug susceptibility in mycobacteria[10]. Besides, there are few reports of transcription factors that directly bind first-line anti-TB drug molecules like rifampicin, and ethambutol (EMB) and regulate the drug resistance of *M. tuberculosis*.

Isothermal titration of calorimetry (ITC) is used for detecting the biothermodynamic and kinetic parameters of biomolecular interactions[11]. Through ITC, we found that a TetR family transcription factor encoded by the *Ms0606* gene in *M. smegmatis* is a novel ethambutol (EMB) binding protein. EMB binds to Ms0606 in a ratio of 1:1, and EMB specifically enhances the binding activity of Ms0606. Furthermore, in the presence of non-lethal dose of EMB, Ms0606 significantly affected the sensitivity of *M. smegmatis* to INH. These results reveal the mechanism of mycobacterial transcription factor-mediated first-line anti-TB drugs EMB and INH combined use to enhance drug efficacy, which provides the theoretical basis for revealing the mechanism by which the transcription factor Ms0606 mediates first-line anti-TB drug resistance.

1 Materials and Methods

1.1 Strains, enzymes, reagents, and plasmids

The *Escherichia coli* BL21(DE3) and the required vehicle for protein expression pET-28a (CM1) were purchased from Novagen; DNA polymerase, dNTPs, restriction enzyme and T4 ligase required for PCR reaction were purchased from TaKaRa Biotech (Dalian); all antibiotic chemicals were purchased from Sigma-Aldrich; Ni^{2+}-nitrilotriacetate purified gels were purchased from GE Healthcare Life Sciences China. The PCR primers used in the experiments were synthesized by Wuhan Tsingke Biotechnology Co., Ltd, and Invitgen Biotechnology Co., Ltd.

1.2 Gene cloning, expression, and purification of Ms0606 protein

The *Ms0606* gene was amplified by using the *M. smegmatis* mc^2155 genome as template, upstream primer was 5′-ATATGAATTCGAGTGA CCGTCAGCCCCCGC-3′, downstream primer was 5′-AGATTCTAGATCATGGCTGCGGTACC CCCC-3′. PCR product and pET-28a (CM1) vector were double digested by *Eco*R I/*Xba* I and then purified with PCR DNA Purification Kit (BioFlux). The *Ms0606* gene was cloned into the pET-28a (CM1) vector and transformed into *E. coli* BL21(DE3) strain, the correct sequenced was selected strain for test expression, test expression to verify the successful preserved glycerol tube.

The stored glycerol tubes were connected to 5 mL Luria-Bertani (LB) medium at a ratio of 1:100, and the activated broth was transferred to 1 L medium containing kanamycin and grown at 37 °C to OD_{600} of approximately 0.8 to 1.0. 0.5 mmol/L isopropyl thiogalactoside (IPTG) was

added to induce protein expression for 4 h, and then the protein was purified by Ni^{2+} column affinity chromatography. First, the cells were centrifuged at 8 000 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), the sonicated supernatant flowed through His-glue beads treated with Ni^{2+} (50 mmol/L $NiSO_4$), and the unbound heteroproteins were washed with 10 to 15 mL Binding buffer (40 mmol/L imidazole; 100 mmol/L NaCl; 20 mmol/L Tris-HCl, pH 8.0). Finally, the target protein was eluted with 15–20 mL of elution buffer (125 mmol/L imidazole; 100 mmol/L NaCl; 20 mmol/L Tris-HCl, pH 8.0) and was detected by SDS-PAGE gel. Proteins with higher purity and concentration were selected for dialysis and stored at –80 °C at 100 L per tube.

1.3 Electrophoretic mobility shift assay (EMSA)

M. smegmatis mc^{2}155 genomic DNA was used as the template, the promoter Ms0606p of target fragment *Ms0606* gene was obtained by PCR amplification. Upstream primer was 5′-GGATCGGGCCGACCCGCACC-3′ and downstream primer was 5′-GGCGCTCCTGAACGTGTCAT-3′, whose 5′ end was labeled fluorescein isothiocyanate (FITC). EMSA experiment was slightly modified on the basis of literature reports[12], 1 L of fluorescently labeled DNA fragments were coincubated with 1–8 L concentration gradient Ms0606 protein in 20 L of EMSA buffer (50 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT), 4 °C reaction for 30 min. For EMSA involving small molecule EMB, 1–4 L of 150–600 μg/mL EMB and INH (control) solutions were mixed with 2 L of Ms0606 protein for 30 min, and then 1 L of labeled DNA fragments were added. The system was supplemented with EMSA buffer to 20 L, and reacted for 4 °C for 30 min. The reaction system was then placed at 150 V for 5% native polyacrylamide gel electrophoresis (40% acrylamide 5 mL, ddH_2O 31 mL, APS 1 μL, TEMED 50 μL) for 1–2 h, finally sent to the typhoon scanner (GE Healthcare) fluorescence scan, and save the results after image processing software analysis.

1.4 Isothermal heat volume titration method (ITC)

The ITC experiment was slightly modified in reference [13]. As shown in Figure 1, the Nano ITC low-volume isothermal calorimeter (TA Instruments, DE) controlled by the ITC Run software is used. Tittrate Ms0606 and titrate EMB were prepared in the same buffer (20 mmol/L Tris-HCl, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, pH 6.5). 20 mol/L His-tagged Ms0606 protein and 30 g/mL EMB solution were added to the sample pool (190 L) and syringe (50 L), 25 injections were measured at 250 r/min and 25 °C to obtain dilution heat. Small molecule EMB was replaced with INH in the control experiment.

1.5 Determination of growth curve of *M. smegmatis* mc^{2}155

To obtain overexpression strains, *Ms0606* coding sequences were amplified using *M. smegmatis* mc^{2}155 genomic DNA as the template, upstream primer was 5′-ATATGAATTCGAGTGACCGTCAGCCCCCGC-3′, downstream primer was 5′-AGATTCTAGATCATGGCTGCGGTACCCCCC-3′, then cloned into the pMV261 vector, and transformed by electroporation into wild-type *M. smegmatis* mc^{2}155 cells. Recombinant strains were selected on 7H10 plates containing 100 μg/mL of kanamycin.

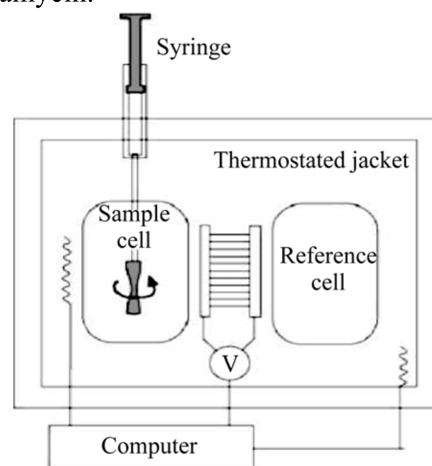

Figure 1 Schematic diagram of the ITC structure.

Then constructed *Ms0606* overexpression strain was transferred from glycerol tube to PA bottle for activation, and then transferred to 100 mL 7H9 medium in a ratio of 1:100 for culture. The medium was cultured at 37 °C until the OD_{600} was 1.5–1.8. An appropriate volume of bacterial liquid was absorbed and transferred to a new 100 mL 7H9 medium. The initial OD_{600} of each strain was the same, with an error of about 0.015. Samples were taken and the initial OD_{600} was recorded as the data of 0 h. Then a series of concentration gradients of isoniazid and ethambutol were added to the culture medium and cultured in a 37 °C shaker at 160 r/min. 2 mL of bacterial solution was taken every 4 h and the absorbance of the bacterial solution OD_{600} was measured by spectrophotometer, samples were taken 3 times each time.

2 Results

2.1 Cloning of the *Ms0606* gene and protein purification

The total genome of *M. smegmatis* mc^2155 extracted by the kit was used as a template, and the *Ms0606* gene fragment was amplified by appropriate PCR conditions. In Figure 2A, a specific band (lane 2) was detected between 925 bp in the ninth band and 421 bp in the tenth band of DNA marker (lane 1), which matched the size of 630 bp of the target fragment, so the amplified fragment was the *Ms0606* gene. PCR verification of recombinant vector pET28a connected with *Ms0606* was shown in Figure 2B, and the fragment size matched the size of the previously amplified *Ms0606* gene (lane 2), indicating that gene *Ms0606* was successfully connected to the pET28a vector. The resulting recombinant vector was tested for protein expression in BL21(DE3), as is shown in Figure 2C, lane 1 is a negative control without IPTG induction, and lane 2–7 are samples after IPTG induction, showing that Ms0606 can be used for subsequent purification.

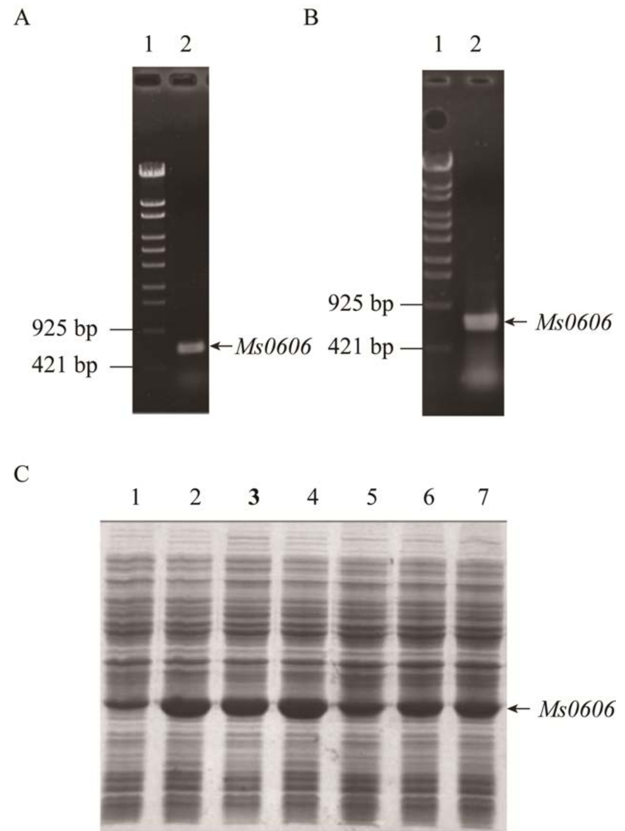

Figure 2 Construction of pET28a-*Ms0606* plasmid and SDS-PAGE analysis of recombinant Ms0606 proteins. A: *Ms0606* amplified by PCR. 1: DNA marker; 2: PCR product. B: PCR validation of pET28a-*Ms0606* vector. 1: DNA marker; 2: PCR validation product of pET28a-*Ms0606*. C: SDS-PAGE determination of Ms0606 expression results. 1: Without IPTG; 2–7: After IPTG induction.

To further investigate the regulatory role of Ms0606, we cloned the gene encoding Ms0606 protein in mycobacteria into the expression vector pET28a, and then expressed Ms0606 protein in the expression strain *E. coli* BL21(DE3), after a gradient concentration of imidazole elution, and obtained a protein with high concentration and purity using nickel column affinity purification. As shown in Figure 3, the target protein His-Ms0606 was detected by SDS-PAGE after elution with 250 mmol/L imidazole (lanes 3–5).

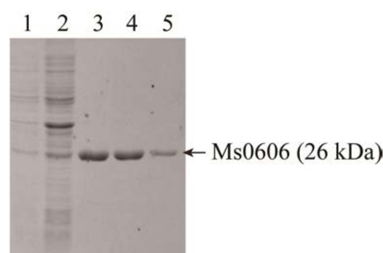

Figure 3 Purification of recombinant Ms0606 proteins. SDS-PAGE was used to detect protein. 1: Upernatant after ultrasonic crushing; 2: Precipitation after ultrasonic crushing; 3–5: 250 mmol/L imidazole elution. The arrow indicates the eluted Ms0606 protein.

To investigate the function of Ms0606, the conserved amino acid sequences of Ms0606 proteins were searched by the NCBI-CDD database. As shown in Figure 4, I found that Ms0606 protein consists of 209 amino acids, the N terminus has the TetR family helix-turn-helix DNA binding domain, and the entire sequence has a canonical AcrR family domain, suggesting that Ms0606 encodes a TetR/AcrR family transcription factor.

2.2 ITC confirmed the interaction between Ms0606 and EMB

To further investigate whether EMB directly targets the Ms0606 protein, ITC was used to detect whether Ms0606 interacts with small molecule EMB. At constant temperature, when 30 g/mL EMB (protein dialysate containing 5% glycerol) was titrated to 20 μmol/L Ms0606, the two substances interact and the heat released is proportional to the amount of binding. As observed in Figure 5, when the EMB solution was injected 25 times into the protein solution of the ITC sample pool, the heat change from the Ms0606 reaction corresponds to a blue peak. When the protein in the pool was saturated with EMB, the heat signal weakens, and finally only the diluted background heat was observed, forming a S-like curve. Using nonlinear regression analysis with Launch Nano Aanlyze software, the results showed that: (1) The action of Ms0606 and EMB is an exothermic reaction; (2) Under our experimental conditions, the binding chemical ratio of EMB and Ms0606 was 1:1 (n=0.94), indicating that one Ms0606 binds one EMB. Following the same method, we titrated Ms0606 with a control small molecule INH, and the experimental results are shown in Figure 5, failing to fit a curve similar to S type, indicating that Ms0606 interacts with EMB, while INH is not.

2.3 EMB promotes the DNA-binding activity of Ms0606

Ms0606, as a transcription factor of the TetR family, usually has an autoregulatory function and specifically recognizes the upstream sites of its own promoter, relevant research results have been published[14]. It has been demonstrated that EMB can physically interact with Ms0606, and we hypothesized that EMB can alter the DNA-binding activity of Ms0606, this hypothesis was further confirmed by EMSA. As shown in Figure 6, when FITC-labeled Ms0606p was incubated with 2 μmol/L of Ms0606 (lanes 1–3), a weak lag band was observed, and clear protein-DNA complex bands were formed with increasing EMB concentration (150–600 μg/mL) (lanes 4–6). Under the same conditions, the negative control molecule INH (150–600 μg/mL; lanes 7–9) had no effect, indicating that EMB can promote the DNA binding activity of Ms0606 on its own promoter.

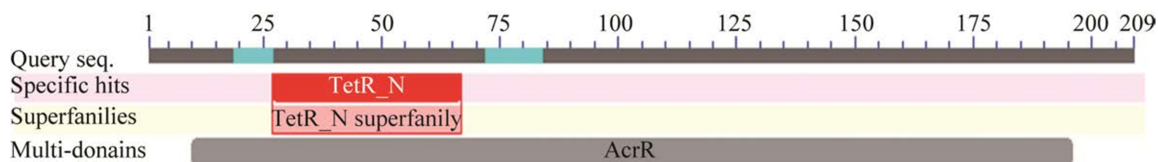

Figure 4 Analysis of the domain structure of Ms0606.

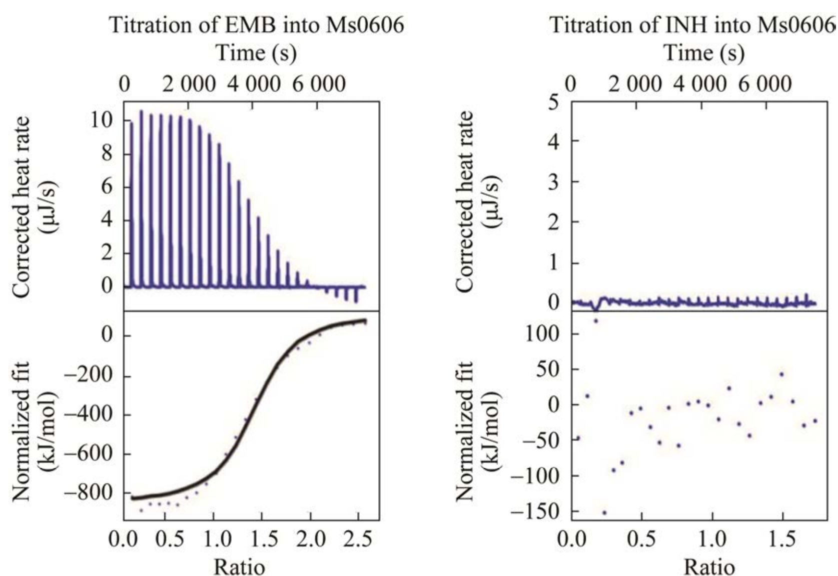

Figure 5 ITC assays for the interaction between Ms0606 and ethambutol.

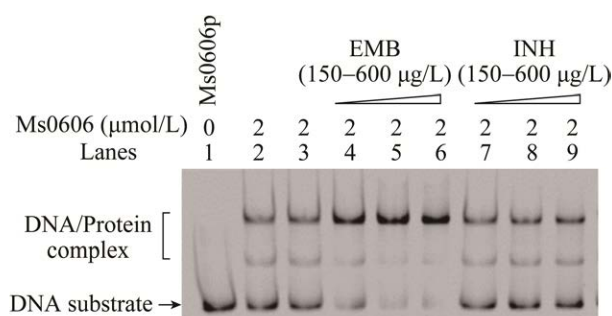

Figure 6 EMSA assays for the DNA-binding activity of Ms0606 stimulated by ethambutol.

2.4 Ms0606 enhances ethambutol mediated isoniazid sensitivity in *M. smegmatis* mc^{2}155

Previous experiments have confirmed that transcription factor Ms0606 regulates the sensitivity of *M. smegmatis* to INH[14], and EMB interacts with Ms0606 protein and promotes the DNA-binding activity of Ms0606. Therefore, we hypothesized that EMB could affect the sensitivity of *Ms0606* overexpressed strain of *M. smegmatis* to INH.

To further verify this hypothesis, we compared the growth curves of wild-type strain (Msm/pMV261) and overexpressed strain (Msm/pMV261-*Ms0606*) with different drug, then sampled every 4 hours and diluted the coating plate for colony count, the growth curve was drawn with the time as the abscissa and the logarithm of the colony forming unit (CFU) as the ordinate.

As shown in Figure 7 (above), the *Ms0606* overexpression strain (Msm/pMV261-*Ms0606*) grew almost the same with an empty control vector (Msm/pMV261) in 7H9 medium without the drug and with 0.2 μg/mL EMB, indicating that 0.2 μg/mL EMB did not have an inhibitory effect on the growth of *M. smegmatis*. However, as shown in Figure 7 (below), in the 7H9 medium containing 5 μg/mL INH, the growth rate of Msm/pMV261-*Ms0606* was lower than that of Msm/pMV261, suggesting that Ms0606 could enhance the sensitivity of *M. smegmatis* to INH. Furthermore, we added 0.2 μg/mL EMB to 7H9 medium containing 5 μg/mL INH, compared with the medium containing only 5 μg/mL INH, the difference between Msm/pMV261-*Ms0606* and Msm/pMV261 was more obvious. Error bars represent three independent biological replicates, P values are calculated by T-distribution test, and asterisks represent significant differences between the two groups (*: P<0.05; **: P<0.01; ***: P<0.001). These results indicate that non-lethal dose EMB in *M. smegmatis* can further improve the sensitivity of Ms0606 overexpressing strain to INH.

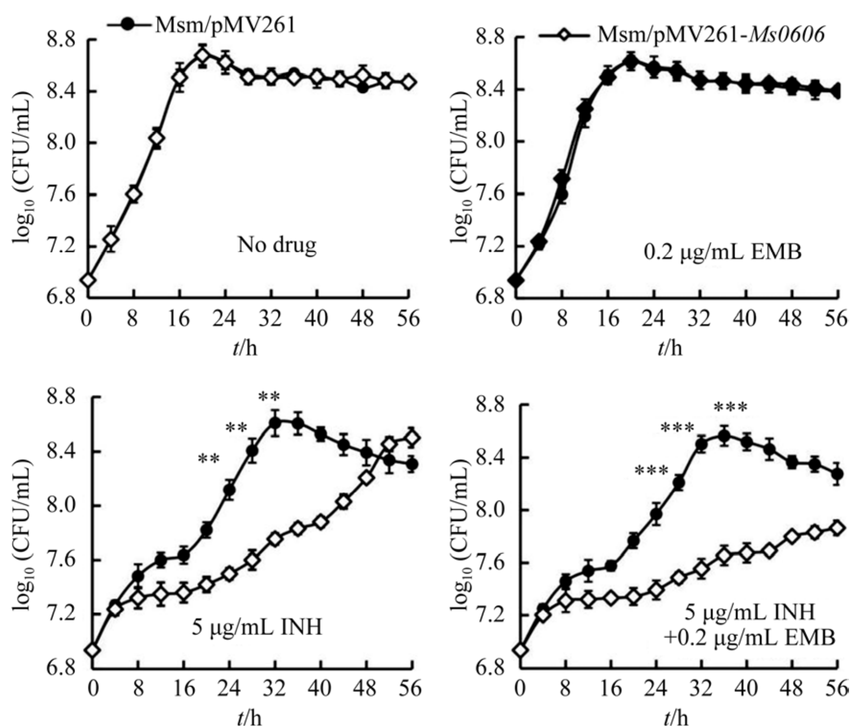

Figure 7 Determination of growth curves of Msm/pMV261 and Msm/pMV261-*Ms0606*.

3 Discussion

The genome of *M. tuberculosis* contains approximately 200 transcription factors[15], transcription factors that interact with drugs are rarely reported, and information on the molecular networks elicited by first-line anti-TB drugs in *M. tuberculosis* is also limited. As far as we know, InbR directly responds to INH, which regulates multiple resistance in mycobacteria by regulating the expression of a large number of potential target genes[12]. In addition, there are few reports of transcription factors that directly bind first-line anti-TB drugs and regulate the multiple resistance of *M. tuberculosis*.

In this study, we found a transcription factor Ms0606 that can directly bind EMB through ITC. The stoichiometry was obtained by fitting analysis, indicating that the force between Ms0606 and EMB is strong and bound in a 1:1 ratio. EMB could promote DNA-binding activity of Ms0606. So EMB can be used as both an anti-TB drug and a signaling molecule, what is the significance of its interaction with Ms0606? I know that the balance between TetR/AcrR family transcription factors regulating target genes and derepressor is mainly controlled by ligand. Bolla et al previously reported that the transcription factor Rv3066 in *M. tuberculosis* binds to ethidium bromide, inhibiting the DNA binding activity of the transcription factor, thus relieving the inhibition of drug efflux pump *mmr* gene and thus increasing drug efflux[16]. The binding of INH and InbR also changed the binding activity of transcription factor and DNA, making the overexpressed strain resistant to multiple drugs[12]. Inspired by this, combined with previous results[14] and recent research, we hypothesize that EMB may act as an inducer and ultimately enhance the sensitivity of mycobacteria to INH .

On the other hand, as for the bactericidal mechanism of EMB, recent studies have resolved the “drug-target” three-dimensional structure of the arabinosyltransferase complexes EmbA-EmbB and EmbC-EmbC[17], revealing that ethambutol ultimately inhibits cell wall synthesis[18] by

targeting EmbA, EmbB and EmbC involved in the synthesis of arabinogalactan AG and lipoarabinomannan LAM. In this study, we found that Ms0606 directly interacts with EMB, this indicates that EMB is not only a drug to control *M. tuberculosis*, but also can be used as a chemical inducer to participate in the formation of drug resistance of other drugs. As shown in Figure 8, a model showing that the EMB-triggering regulatory pathway through Ms0606 enhances the INH sensitivity of *M. smegmatis*. Previous studies have confirmed that Ms0606 negatively regulates the sensitivity of *M. smegmatis* to INH[14]. EMB can enhance the DNA-binding activity of Ms0606 and make the mycobacterial cells more sensitive to INH. The purple oval represents repressor protein Ms0606, yellow semicircle represents EMB molecular, it can bind with Ms0606 and enhances its DNA binding activity. Eventually making cells more sensitive to INH radicals (purple small bend bar). The study of its regulatory mode will explain the new mechanism of transcription factor-mediated multiple drug resistance in mycobacteria.

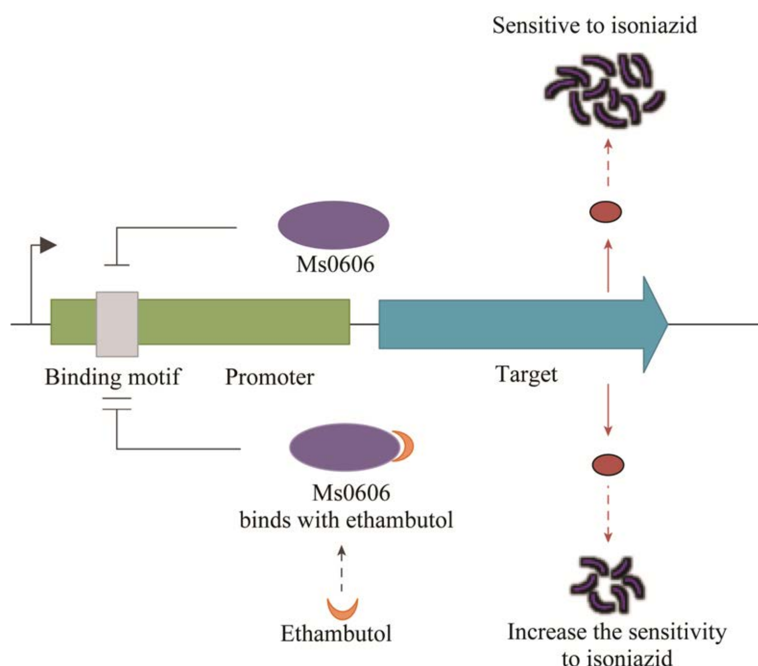

Figure 8 Schematic diagram of the regulatory model.

4 Conclusion

In conclusion, EMB as the main first-line anti-TB drug, is widely used in clinical practice. This study demonstrates that EMB, besides acting as an antimicrobial agent, also acts as an inducer to promote the killing effect of another first-line drug INH on mycobacteria. This finding provides new examples of anti-TB drugs directly binding transcription factor proteins and enhances our understanding of drug-resistance-related regulatory mechanisms and signaling pathways in bacteria.

参考文献

[1] NIKAIDO H. Multidrug resistance in bacteria[J]. Annual Review of Biochemistry, 2009, 78: 119-146.

[2] VILCHÈZE C, WEISBROD TR, CHEN B, KREMER L, HAZBÓN MH, WANG F, ALLAND D, SACCHETTINI JC, JACOBS WR Jr. Altered NADH/NAD$^+$ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria[J]. Antimicrobial Agents & Chemotherapy, 2005, 49(2):708-720.

[3] CUTHBERTSON L, NODWELL JR. The TetR family of regulators[J]. Microbiology and Molecular Biology Reviews, 2013, 77(3): 440-475.

[4] LI M, GU R, SU CC, ROUTH MD, HARRIS KC, JEWELL ES, MCDERMOTT G, YU EW. Crystal structure of the transcriptional regulator AcrR from *Escherichia coli*[J]. Journal of Molecular Biology, 2007, 374(3): 591-603.

[5] YU Z, REICHHELD SE, SAVCHENKO A, PARKINSON J, DAVIDSON AR. A comprehensive analysis of structural and sequence conservation in the TetR family transcriptional regulators[J]. Journal of Molecular Biology, 2010, 400(4): 847-864.

[6] SAENGER W, ORTH P, KISKER C, HILLEN W, HINRICHS W. The tetracycline repressor-a paradigm for a biological switch[J]. Clinical Journal of the American Society of Nephrology, 2000, 39(12): 2042-2052.

[7] TAKEUCHI K, IMAI M, SHIMADA I. Conformational equilibrium defines the variable induction of the multidrug-binding transcriptional repressor QacR[J]. Proceedings of the National Academy of Sciences of the United States of America, 2019, 116(40): 19963-19972.

[8] WANG K, SYBERS D, MAKLAD HR, LEMMENS L, LEWYLLIE C, ZHOU X, SCHULT F, BRÄSEN C, SIEBERS B, VALEGÅRD K, LINDÅS AC, PEETERS E. A TetR-family transcription factor regulates fatty acid metabolism in the archaeal model organism *Sulfolobus acidocaldarius*[J]. Nature Communications, 2019, 10(1): 1542.

[9] SINGH AK, MANJASETTY B, BALASUBRAMANI GL, KOUL S, KAUSHIK A, EKKA MK, SINGH V, KUMARAN S. Crystal structure of Fad35R from *Mycobacterium tuberculosis* H37Rv in the apo-state[J]. PLoS One, 2015, 10(5): e0124333.

[10] YANG M, JIA SH, TAO HL, ZHU C, JIA WZ, HU LH, GAO CH. Cd(II)-binding transcriptional regulator interacts with isoniazid and regulates drug susceptibility in mycobacteria[J]. The Journal of Biochemistry, 2021, 169(1): 43-53.

[11] VELÁZQUEZ-CAMPOY A, OHTAKA H, NEZAMI A, MUZAMMIL S, FREIRE E. Isothermal titration calorimetry[J]. Current Protocols in Cell Biology, 2004, Chapter 17: Unit 17.8. doi: 10.1002/0471143030.cb1708s23.

[12] YANG M, GAO CH, HU J, ZHAO L, HUANG Q, HE ZG. InbR, a TetR family regulator, binds with isoniazid and influences multidrug resistance in *Mycobacterium bovis* BCG[J]. Scientific Reports, 2015, 5: 13969.

[13] LI W, CUI T, HU L, WANG Z, LI Z, HE ZG. Cyclic diguanylate monophosphate directly binds to human siderocalin and inhibits its antibacterial activity[J]. Nature Communications, 2015, 6: 8330.

[14] 朱晨. 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(英文)[J]. 微生物学报, 2021, 61(8): 2530-2544.
ZHU C. A TetR-like regulator negatively regulates isoniazid resistance in *Mycobacterium smegmatis*[J]. Acta Microbiologica Sinica, 2021, 61(8): 2530-2544 (in Chinese).

[15] COLE ST, BROSCH R, PARKHILL J, GARNIER T, CHURCHER C, HARRIS D, GORDON SV, EIGLMEIER K, GAS S, BARRY CE 3rd, TEKAIA F, BADCOCK K, BASHAM D, BROWN D, CHILLINGWORTH T, CONNOR R, DAVIES R, DEVLIN K, FELTWELL T, GENTLES S, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence[J]. Nature, 1998, 393(6685): 537-544.

[16] BOLLA JR, DO SV, LONG F, DAI L, SU CC, LEI HT, CHEN X, GERKEY JE, MURPHY DC, RAJASHANKAR KR, ZHANG QJ, YU EW. Structural and functional analysis of the transcriptional regulator Rv3066 of *Mycobacterium tuberculosis*[J]. Nucleic Acids Research, 2012, 40(18): 9340-9355.

[17] ZHANG L, ZHAO Y, GAO Y, WU L, GAO R, ZHANG Q, WANG Y, WU C, WU F, GURCHA SS, VEERAPEN N, BATT SM, ZHAO W, QIN L, YANG X, WANG M, ZHU Y, ZHANG B, BI L, ZHANG X, et al. Structures of cell wall arabinosyltransferases with the anti-tuberculosis drug ethambutol[J]. Science, 2020, 368(6496): 1211-1219.

[18] TAKAYAMA K, ARMSTRONG EL, KUNUGI KA, KILBURN JO. Inhibition by ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*[J]. Antimicrobial Agents and Chemotherapy, 1979, 16(2): 240-242.