Molecular mechanism of the synergistic activity of ethambutol and isoniazid against *Mycobacterium tuberculosis*

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Received for publication, March 1, 2018, and in revised form, August 26, 2018 Published, Papers in Press, September 5, 2018, DOI 10.1074/jbc.RA118.002693

Isoniazid (INH) and ethambutol (EMB) are two major first-line drugs for managing tuberculosis (TB), caused by the microbe *Mycobacterium tuberculosis*. Although co-use of these two drugs is common in clinical practice, the mechanism for the potential synergistic interplay between them remains unclear. Here, we present first evidence that INH and EMB act synergistically through a transcriptional repressor of the *inhA* gene, the target gene of INH encoding an enoyl-acyl carrier protein reductase of the fatty acid synthase type II system required for bacterial cell wall integrity. We report that EMB binds a hypothetical transcription factor encoded by the *Rv0273c* gene, designated here as EtbR. Using DNA footprinting, we found that EtbR specifically recognizes a motif sequence in the upstream region of the *inhA* gene. Using isothermal titration calorimetry and surface plasmon resonance assays, we observed that EMB binds EtbR in a 1:1 ratio and thereby stimulates its DNA-binding activity. When a nonlethal dose of EMB was delivered in combination with INH, EMB increased the INH susceptibility of cultured *M. tuberculosis* cells. In summary, EMB induces EtbR-mediated repression of *inhA* and thereby enhances the mycobacterial effect of INH. Our findings uncover a molecular mechanism for the synergistic activity of two important anti-TB drugs.

This work was supported by National Key R&D Program of China Grant 2017YFD0500300, National Natural Science Foundation of China Grants 31730005 and 81471996, Fundamental Research Funds for the Central Universities Grants 2662016PY090 and 2662016QD007, and the Chang Jiang Scholars Program (to Z.-G. H.). The authors declare that they have no conflicts of interest with the contents of this article. This article contains Tables S1–S4 and Figs. S1–S12.
study presents a novel model for the enhanced killing of M. tuberculosis using a drug combination.

Results

Noninhibitory concentration of EMB increases the INH sensitivity of M. tuberculosis

Our studies originated from a determination of the minimal inhibitory concentrations (MICs) of INH for M. tuberculosis H37Ra in the presence of a low concentration of EMB. As shown in Table 1 and Fig. S1, in the absence of EMB in the medium, the determined MIC of INH is 0.025 μg/ml, which is very close to the value reported previously (10, 11). Interestingly, the MIC (0.0125 μg/ml) of INH in combination with 0.2 μg/ml EMB was found to be 2 times lower than the MIC of INH alone. Essentially, 0.2 μg/ml EMB did not have an inhibitory effect on the growth of M. tuberculosis under the experimental conditions (Fig. S2). This observation suggests that the noninhibitory concentration of EMB could increase the sensitivity of M. tuberculosis H37Ra to INH.

The expression level of EtbR affects the INH sensitivity of M. tuberculosis

We further found that the enhancing effect of EMB for INH was related to the expression level of Rv0273c (EtbR). As shown in Table 1 and Fig. S1, when the plasmid pMV261 was used to overexpress EtbR in M. tuberculosis H37Ra, the recombinant mycobacterial strain obtained a lower MIC for INH (0.0125 μg/ml) than the WT strain, indicating that the expression of EtbR increased the mycobacterial sensitivity to INH. We further determined the MIC of the etbR overexpression strain for INH in the presence of a low concentration of EMB (0.2 μg/ml). Interestingly, the value (0.003125 μg/ml) was 4 times lower than that from the WT strain (above 0.0125 μg/ml). By contrast, the MIC of the etbR-knockout strain for INH in the presence of a low concentration of EMB was not changed (Table 1 and Fig. S1). Therefore, increasing the expression of EtbR could further improve the INH sensitivity of M. tuberculosis triggered by EMB.

EtbR interacts with the upstream regulatory sequence of inhA

The expression of EtbR could improve the EMB-triggered INH sensitivity, suggesting that the enhancing mechanism of EMB was linked to the regulatory role of EtbR. To test this idea, we first examined whether EtbR directly interacts with the upstream DNA sequence of the target gene of INH, inhA.

The etbR gene encodes a 206-residue protein containing a typical TetR N-terminal helix-turn-helix domain within an AcrR domain (Fig. 1A). Thus, EtbR belongs to the TetR/AcrR family of transcription factors. Strikingly, both EtbR and its operon structure are highly conserved in several mycobacterial species, including M. tuberculosis H37Rv, Mycobacterium bovis BCG, and Mycobacterium smegmatis (Fig. S3). We used an electrophoretic mobility shift assay (EMSA) to examine the binding of the purified EtbR protein to the upstream region of inhA. When 3 nm upstream DNA substrates, inhAp, and a control DNA fragment (Rv0275cp) were co-incubated with increasing amounts of EtbR (1–8 μM), clear DNA–protein complex bands could be observed when 8 μM EtbR was co-incubated with inhAp (Fig. 1B, lanes 2–5), but not Rv0275cp. This indicated that EtbR could bind with the upstream DNA fragment of the inhA gene. In addition, EtbR could also bind well with the upstream DNA fragment of etbR itself (Fig. S4).

We subsequently characterized the motif sequence recognized by EtbR. Both upstream DNA fragments of etbR and inhA genes were subjected to DNase I footprinting assays. As shown in Figs. S5 and S6, the protected DNA region by the EtbR protein was found within two DNA fragments, respectively, and a motif sequence, 5’-GAATGTGAAGCAGCAGACAG-3’ (the conserved bases are underlined), was thereafter identified (Fig. 1C). Furthermore, a mutagenesis analysis could clearly confirm the significance of the motif sequence for the recognition of EtbR (Fig. S5 and S6).

ChIP assays were conducted to further confirm the binding of EtbR with these two DNA fragments in vivo in M. bovis BCG. As shown in Fig. 1D, both DNA fragments could be specifically recovered by EtbR antibody in the WT strain (Fig. 1D, left) but not in the etbR-deleted mutant strains (Fig. 1D, right). By contrast, the promoter of a negative control, Rv0275cp, could not be recovered under similar conditions. Therefore, EtbR specifically interacted with the upstream DNA sequence of inhA operon both in vitro and in mycobacteria.

EtbR acts as a repressor and inhibits expression of inhA

EtbR is a hypothetical TetR family transcription factor, suggesting that it functions as a repressor. To test this assumption, we constructed an etbR-deleted mutant strain (Fig. S7) and an etbR-overexpressing strain in M. bovis BCG. qRT-PCR assays were subsequently used to compare the expression difference of the inhA gene between these mycobacterial strains. As shown in Fig. 2A, inhA expression was significantly up-regulated in the etbR-deleted strain compared with the WT strain. Consistently, inhA expression was clearly down-regulated in the etbR-overexpressing strain (Fig. 2B). These results indicate that EtbR acted as a repressor and negatively regulated the expression of inhA.

Table 1

Determination of the MIC of anti-TB drugs

The anti-mycobacteria activity/effect of the anti-TB drugs was measured by the REMA. The results were carried out with three independent biological replicates. MIC for M. tuberculosis H37Ra/pMV261-etbR is significantly different (p < 0.001, two-tailed Student’s t test) between INH and INH + 0.2 μg/ml EMB.

| Drugs         | M. tuberculosis H37Ra/pMV261 | M. tuberculosis H37Ra/pMV261-etbR | BCG/pMindD | BCG/etbR::hyg/pMindD | BCG/etbR::hyg/pMindD-etbR |
|---------------|------------------------------|-----------------------------------|------------|-----------------------|---------------------------|
| INH           | 0.025                        | 0.0125                             | 0.025      | 0.05                  | 0.025                     |
| EMB           | 0.25                         | 0.25                               | 0.25       | 0.25                  | 0.25                      |
| INH (+0.2 EMB)| 0.0125                       | 0.003125                           | 0.0125     | 0.05                  | 0.0125                    |
Using β-gal as the reporter, a series of promoter-lacZ reporter plasmids was further constructed (Fig. 2C) to examine the regulatory effects of EtbR on inhA expression. These plasmids were transformed into M. smegmatis, generating a series of reporter strains, and subjected to the assays for their β-gal activities. As shown in Fig. 2C, the positive control promoter hsp60 strongly promoted lacZ expression, but no expression of lacZ for null promoter was observed. Thus, the reporter system functioned well. The promoter activity of either etbR or inhA promoters alone was shown to be robust, but both activities were significantly reduced in the presence of etbR (Fig. 2C). Notably, deleting the EtbR binding site within either the Rv0273cp or inhAp reporter constructs eliminate the inhibition (Fig. 2C). Interestingly, we found that the relative gene expression level of etbR is ~6-fold higher in inhAp-etbR-lacZ fusion than Rv0273cp-etbR-lacZ fusion (Fig. S8), suggesting that the auto-repression is more efficient. These results indicate that EtbR repressed the expression of the reporter gene, and they were consistent with the qRT-PCR data above. Therefore, EtbR functioned as a repressor and inhibited the expression of inhA.

**EMB physically interacts with EtbR**

We further examined whether EMB directly targets the EtbR protein. First, an isothermal titration calorimetry (ITC) assay was used to detect the potential binding of EMB with EtbR. Fig. 3A shows the raw data for titration of EMB against EtbR, and it indicates that the interaction is exothermic. Furthermore, results showed that the binding stoichiometry between EMB against EtbR was 1:1 (n = 1,008), and the binding affinity of the interaction (K_d) was 1.06 μM under our experimental conditions, which suggested a strong binding affinity between them. By contrast, no binding for INH was detected under similar experimental conditions (Fig. S9A). A further surface plasmon resonance (SPR) assay confirmed that EtbR specifically recognized EMB (Fig. 3B). A corresponding increase in response was observed when an increasing amount of EMB (60–480 μg/ml) was passed over the EtbR-immobilized CM5 chip. In particular, when 480 μg/ml EMB was passed over the chip, more than 150 resonance units (RU) was observed, indicating effective binding of EMB with EtbR (Fig. 3B). By contrast, neither INH nor GTP could interact with EtbR (Fig. S9B). Taken together, both ITC and SPR assays suggested that EMB could physically interact with EtbR.

**EMB stimulates the DNA-binding activity of EtbR and enhances its repressive regulation**

EtbR specifically recognizes the upstream site of the inhA gene, and EMB could physically interact with EtbR, suggesting that EMB would regulate the DNA-binding activity of EtbR. An EMSA could confirm this hypothesis. As shown in Fig. 3C, 2 μM EtbR alone could shift the mobility of the upstream regulatory DNA substrates of inhA. A stepwise increment of DNA–protein complexes (Fig. 3C, lanes 4–6) was clearly observed when adding increasing amounts of EMB (150–600 μg/ml) into the reactions. The DNA was 50% bound at 6.8 μM and 2.0 μM EtbR in the absence and presence of EMB, respectively, indicating that EMB enhances EtbR–DNA binding (Fig. S9C). Here, the amount of EMB is higher than the amount of EMB in the ITC assay, as the ITC assay required an optimized EMB concentration to resolve the K_d constant. EMSA is a qualitative assay and requires running a polyacrylamide gel to show a result. Also, DNA substrate was added in EMSA; thus, the solution content is different from that in ITC. Higher amounts of EMB were used to ensure complete EtbR–EMB complex formation. By contrast, no similar regulatory role for INH was detected under the same conditions (Fig. 3C, lanes 7–9).
we replaced full-length inhAp with the short binding motif inhAp1, similar results were obtained (Fig. S10). This phenomenon indicates that EMB could specifically stimulate the DNA-binding activity of EtbR.

SPR experiments further confirmed this conclusion. As shown in Fig. 3D, when increasing concentrations of the EtbR protein (0.5–2 μM) passed over the inhAp-immobilized streptavidin (SA) chip, corresponding increases in response values were observed (Fig. 3D, left). When a fixed concentration of EtbR (0.5 μM) was co-incubated with increasing concentrations of EMB (150 μg/ml to 600 μg/ml) and passed over the chip, corresponding increases in response values could be clearly observed (Fig. 3D, right), which were much higher than the values with EtbR alone. No obvious change in the response value was observed when either unrelated Rv0275c protein or INH was passed over the chip. These results indicated that EMB enhances the binding of EtbR to inhAp.

EMB stimulated DNA-binding activity of the repressor, EtbR, implying that EMB enhanced its negative regulation. A further β-gal assay confirmed this assumption. As shown in Fig. 4A, the activity of the inhA promoter was good, and the introduction of EtbR significantly repressed the expression levels. Compared with the lack of EMB induction, we observed that the galactosidase activities of inhAp- etbR-lacZ fusion significantly decreased (Fig. 4A) when 10 μg/ml EMB was added into the medium for induction. As a comparison, the galactosidase activity of Rv0275cp- etbR-lacZ fusion does not have such an effect (Fig. 4A), as EtbR does not bind to Rv0275cp in vivo (Fig. 1D). This result indicates that EMB obviously enhanced the repression of EtbR. A qRT-PCR assay was then utilized to confirm that EMB could inhibit the expression of inhA in mycobacteria. When M. bovis BCG grew in 7H9 medium up to an A600 of 1.2, an increasing amount of EMB (0, 0.5, and 1 μg/ml) was added into the culture for induction, respectively. As shown in Fig. 4B, the expression of inhA in M. bovis BCG was 2-fold down-regulated in the presence of 1 μg/ml EMB if compared with that in the absence of EMB induction. Meanwhile, the expression of mabA, which is located upstream of inhA (Fig. 1C), was somehow affected by EMB (Fig. S11). Taken together, our results showed that EMB stimulated the DNA-binding activity of EtbR and enhanced its inhibition on inhA expression in mycobacteria.
Our MIC data (Table 1) suggested that the expression level of EtbR was correlated with EMB-stimulated INH sensitivity. Using *M. bovis* BCG as a model strain, we further confirmed this observation by determining the mycobacterial growth in the presence of INH with various sublethal EMB levels (0, 0.15, and 0.2 μg/ml EMB; Fig. 5 and Fig. S12). As shown in Fig. 5 (top left), in the absence of drug, the etbR overexpression strain (*M. bovis* BCG/pMV261- etbR) in the 7H9 medium grew almost the same with an empty control vector (*M. bovis* BCG/ pMV261). However, when 0.02 μg/ml INH was added into the medium, the growth of etbR overexpression strain was slightly slower than that of the control strain, indicating that enhancement of etbR expression level increased the INH sensitivity of *M. bovis* BCG. This result was also consistent with the observation above that EtbR inhibited the expression of the target gene of INH, *inhA*. Subsequently, we confirmed that a nonlethal dose of EMB could further improve INH sensitivity of the etbR overexpression strain. As shown in Fig. 5 (bottom) and Fig. S12A, when a nonlethal dose of EMB (0.15 or 0.02 μg/ml) along with INH (0.02 μg/ml) was added into the medium, the growth of the etbR overexpression strain became significantly slower than that of the WT control strain (p < 0.001). The similar phenotype also appears in *M. tuberculosis* H37Ra strain (p < 0.01) (Fig. S12B). This result indicates that EMB further improved the INH sensitivity of the etbR-overexpressing mycobacterial strain. Therefore, etbR overex-
expression enhanced INH inhibition on the mycobacterial growth. A nonlethal dose of EMB could further improve the inhibitory effect.

**Discussion**

Little is known about the interplay between several first-line anti-TB drugs. In the current study, we characterized a TetR family regulator, EtbR encoded by Rv0273c in *M. tuberculosis*, as a novel EMB-binding protein that may play a role in the interplay of two anti-TB first-line drugs, INH and EMB. EtbR functions as a transcriptional repressor for the expression of *inhA*, a drug target of INH. EMB can enhance the DNA-binding activity of EtbR and reduce InhA levels to make the mycobacterial cells more sensitive to INH. Our findings support a model in which EMB acted as an inducing compound and enhanced the inhibition of EtbR on the expression of *inhA* (Fig. 6). This would result in the reduction of enoyl-ACP reductase of the fatty acid synthase type II system, an essential cell wall mycolic acid biosynthesis enzyme; therefore, INH could kill the mycobacterium easily.

In this study, EtbR-mediated INH sensitivity was attributed to the level changes of drug target gene expression. In general, intrinsic drug sensitivity or resistance in *M. tuberculosis* has been attributed to a combination of a highly impermeable mycolic acid–containing cell wall and active drug efflux mechanisms (12). In recent years, the regulation of cell wall metabolism and drug efflux genes was observed to affect mycobacterial drug sensitivity. For example, WhiB7 is responsible for the tolerance of diverse antibiotics in *M. tuberculosis* (13). Rv3066 is a TetR-like transcriptional regulator that controls the expression of the Mmr efflux system, and its regulatory activity is modulated by the direct binding of ethidium, which leads to derepression of Mmr and promotes drug resistance (14). In our previous

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**Figure 4. Effects of EMB on the transcriptional activity and expression level of EtbR.** A, β-gal activity assays for regulation of EtbR in the absence (No drug) or presence of EMB. The reporter cassettes are identical to that in Fig. 2. Different significances of indicated groups were calculated and are shown by p value and asterisks. B, qRT-PCR assays for *inhA* expression patterns in *M. tuberculosis* WT strain under various concentrations (0–1 μg/ml) of EMB for 24 h. The data were analyzed as in Fig. 2. Error bars, S.D. of replicates. Asterisks represent significant difference (***, p < 0.001, two-tailed Student’s t test) between two groups.

**Figure 5. Assays for the effects of EtbR on INH resistance in M. bovis BCG.** Growth curves of *M. bovis* BCG/pMV261 (control) and *M. bovis* BCG/pMV261-etbR (etbR-overexpressing) strains in the presence or absence of drug were determined. Error bars, S.D. of three biological replicates. Asterisks represent significant difference (*, p < 0.05; **, p < 0.01; ***, p < 0.001, two-tailed Student’s t test) between two groups.
Synergistic action of isoniazid and ethambutol

Figure 6. Model showing that the EMB-triggering regulatory pathway through EtbR enhances the INH sensitivity of *M. tuberculosis*. The solid orange disc with a notch represent EtbR, the repressor protein to both etbR and inhA. The solid green triangle represents the EMB molecule; it can bind with EtbR and enhances its DNA-binding activity, thus down-regulating the expression of *inhA* (color diamond bar, *inhA* operon). Less *InhA* (short blue round bars) makes cells more sensitive to INH radicals (small purple bent bars).

In summary, INH and EMB are major first-line anti-TB drugs, and their combination has been widely adopted in clinical practice for the treatment of TB. However, the mechanism for a potential synergistic action between these two drugs has not yet been documented. This study demonstrated that EMB itself was a killing drug for the control of *M. tuberculosis* and also acted as an enhancing chemical agent for the killing effect of another drug, INH. This finding uncovered a novel mechanism for the synergistic action of these two anti-TB drugs.

**Experimental procedures**

**Strains, enzymes, plasmids, and reagents**

*Escherichia coli* DH5α, BL21 (A DE3) cells, and pET28a (Table S1) were purchased from Novagen (Darmstadt, Germany) and were used to clone *M. tuberculosis etbR* (Rv0273c) and express EtbR protein. DNA polymerase, dNTPs, restriction enzymes, modification enzymes, T4 ligase, and all antibiotics were obtained from TaKaRa Biotech (Shiga, Japan). Ni²⁺-NTA–agarose, sensor chip CM5 was purchased from Qiagen.
Synergistic action of isoniazid and ethambutol

(Hilden, Germany). The 7H9/7H10 medium and oleic acid–albumin–dextrose–catalase (OADC) enrichment, which were used for growing *M. tuberculosis* H37Rv, were purchased from BD Biosciences. INH, EMB, and rifampicin were purchased from Sigma-Aldrich. Antiserum was obtained from the Wuhan Animal Center of the Chinese Academy of Sciences (Wuhan, China).

MIC determination

Determination of the MIC of INH to *M. tuberculosis* H37Rv was performed by a resazurin microtiter assay (REMA) using a 96-well microplate as described previously (21). The mycobacteria were adjusted to a density of 1 × 10⁶ cfu/ml in fresh culture broth. Finally, the bacterial suspensions were inoculated into all wells of a 96-well microtiter plate containing final concentrations of INH (0.1–0.00625 μg/ml), EMB (2–0.03 μg/ml), and EMB (0.2 μg/ml) + INH (0.1–0.00625 μg/ml), and growth controls containing no drugs were also included. The 96-well plates, covered with lids, placed in a plastic bag, were incubated at 37 °C for 2 days. A change in color from blue to pink, indicating bacterial growth, showed the reduction of resazurin caused by bacterial metabolism, which was observed after 2 days of incubation. The MIC was expressed as the lowest concentration of the drug that inhibited mycobacteria growth or prevented change in color of the resazurin from blue to pink on a REMA.

Expression and purification of recombinant proteins

The *etbR* gene was amplified by PCR using a pair of primers (5’-ATAAAGAATTCAATGCGGCATTTCCGAGCAG-3’ and 5’-CAGCTCTAGAGGTTCAATTGTGAGTCCCTCCT-3’; Table S2). The amplification program was as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 30 s, 72 °C for 2 min, and a final extension of 72 °C for 8 min, respectively. The PCR products were purified with the BioFlux PCR DNA purification kit (BioFlux). DNA substrates (<80 bp), just as Rv0273cp1–4, were purchased from Invitrogen (Shanghai, China). Other PCR primers or oligosubstrate sequences are given in Table S2. Those used in footprinting or competition assays were labeled with 5’-FITC, as indicated in under “Results” or in the figure legends. In each assay, DNA substrates were co-incubated at 25 °C for 30 min with increasing concentrations of EtbR proteins (0–8 μM) in a total volume of 20 μl of EMSA buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, and 50 mM NaCl) with or without EMB (150–600 μg/ml). The mixtures were then subjected to 5% native PAGE containing 0.5× Tris borate-EDTA buffer at 150 V for 1 h. Images of gels were acquired using a Typhoon scanner (GE Healthcare).

Construction of the *etbR* recombinant strains of mycobacteria

The *etbR* PCR products were digested by the corresponding restriction endonucleases and then cloned into a pMV261 vector (22) and transformed into *M. bovis* BCG and *M. tuberculosis* H37Rv to generate *etbR* overexpression strains, and the *etbR* genes were cloned into a pMindD vector (17) for constructing a complementary strain. Knockout of the *etbR* in *M. bovis* BCG was performed as described previously (20). Briefly, a pMind-derived suicide plasmid carrying a hygromycin resistance gene was constructed, and a sacB gene was inserted to confer sensitivity to sucrose as a negative selection marker. The recombinant plasmid pMind-*etbR* was electroporated into *M. bovis* BCG—competent cells and selected on 7H10 medium containing 100 mg/ml hygromycin and 4% sucrose. Genomic DNA from allelic exchange mutants in which the *etbR* gene had been deleted was identified by restriction digestion and confirmed by PCR analysis and Southern blotting.

ChIP

ChIP was performed as described previously (23). In general, *M. bovis* BCG WT and *etbR*-deleted strain cells were grown in 100 ml of 7H9 medium up to an *A₆₀₀* of 1.0 and then fixed with 1% formaldehyde for 20 min and terminated with 0.125 mM/ml glycine for 5 min. Cross-linked cells were harvested and resuspended in 1 ml of Tris-buffered saline with Tween 20 and Triton X-100 (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 0.1% Triton X-100). The sample was sonicated on ice, and the average DNA fragment size was determined to be ~0.5 kb. A 100-μl sample of the extract was saved as the input fraction, whereas the remaining 900 μl was incubated with 10 ml of antibodies against EtbR or preimmune serum under rotation for 3 h at 4 °C. The complexes were immunoprecipitated with 20 μl of 50% protein A–agarose for 1 h under rotation at 4 °C. The immunocomplex was recovered by centrifugation.
and resuspended in 100 μl of TE (20 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% SDS). Cross-linking was reversed by a water bath at 65 °C for 6 h. The DNA samples of the input and ChIP were purified, resuspended in 50 μl of TE, and analyzed by PCR with Platinum Taq (Invitrogen) using two pairs of primers (primers of inhAp and Rv0273cp as EMSA; Rv0275cp, 5’-CAGCTGTAGGTCGGATCGA-3’ and 5’-ACGGATCATCTTGACACTCG-3’; Table S3). Each experiment was performed in duplicate and repeated twice. The application protocol included one denaturation step of 5 min at 95 °C and then 25 cycles of 1 min at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The PCR products were then subjected to 1.5% agarose gel electrophoresis containing 1× Tris acetate-EDTA buffer at 100 V for 30 min.

**Determination of mycobacterial growth and the effect of antibiotics**

A mycobacterial growth assay was performed as described previously (20). *M. bovis* BCG WT and etbR-overexpressing strains were grown in Middlebrook 7H9 medium (supplemented with 10% OADC, 0.05% Tween 80, and 0.2% glycerol) containing 30 μg/ml Kan for a week. Cells were cultured to an A600 between 1.5 and 1.8, and each culture was diluted (1:100) in 100 ml of fresh 7H9 broth. The cultures (A600 ~0.25) were then allowed to grow further at 37 °C with shaking at 160 rpm for an extra week. Aliquots were taken during this period, and the growth was measured by the numeration of cfu. Each analysis was performed in triplicate.

**ITC analysis**

The ITC binding experiments was performed as described previously (24). ITC measurements were carried out at 25 °C with a Nano ITC Low Volume isothermal calorimeter (TA Instruments, New Castle, DE) controlled by ITCRun software. The titrant (EtbR) and titrate (EMB) were prepared in the same buffer (20 mM Tris base, 100 mM NaCl, 5 mM MgCl2, pH 6.5). EtbR (20 μM) and EMB solution (30 μg/ml) were added to the sample cells (190 μl) and the syringe (50 μl). In control experiments, the EMB solution was titrated into the buffer in sample cells to obtain the heat of dilution. The value of the heat of dilution was then subtracted from the experimental curve in the final analysis. All of the titration curves were fitted to the independent-site binding model. Background titrations of titrant, EMB into buffer, and H2O into protein sample were also collected, and data were baseline-resolved using the appropriate background titration.

**Quantitative real-time PCR assays**

Extraction of mRNA from WT and recombinant *M. bovis* BCG strains (BCG/pMV261, etbR::hyg, and BCG/pMV261-etbR) and real-time PCR analysis were subsequently carried out as described previously (25). Each PCR (20 μl) contained 10 μl of 2×SYBR Green Master Mix Reagent (Applied Biosystems), 1.0 μl of cDNA samples, and 200 nM gene-specific primers (etbR, 5’-CAGAAAGCCGATCAGCCTGCA-3’ and 5’-TCGGGACTGACAATCACT-3’; maba, 5’-TACAGGGCGGTAGAAAGAGCA-3’ and 5’-CGGTGAGGTTGGCCTGAT-3’; inhA, 5’-TCGGGCTATTGCTATGCTTC-3’ and 5’-GGGACCCGTCATCGTTGAG-3’; Table S4). The reactions were performed in a Bio-Rad CFX RT-PCR machine under the following thermocycling conditions: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Amplification specificity was assessed by conducting melting curve analysis. Expression levels of different genes were normalized to the levels of σA gene transcripts. The degrees of expression change were calculated using the 2^−ΔΔCt method (26). Average relative expression levels and S.D. values were determined from three independent experiments.

**β-gal activity assays**

β-gal activity experiments were performed in *M. smegmatis* MC2 155 by constructing a series of operon-lacZ fusion plasmids derived from pMV261 (27). Target gene promoter fragments of Rv0273p and inhAp were amplified by PCR using two pairs of primers (Rv0273p, 5’-AAATATGCCGCTGACCTGCTGCAATCGGTAAGTTT-3’ and 5’-AGACTCTAGACTGGGCACCTCCCTGGGAACA-3’; inhAp, 5’-GACCAGATTCTCGAAGTGTTGTAGTGAATCACCCGAC-3’ and 5’-AATTTCCTAGATGCTGCTTGTTGTTGCTAGTG-3’; Table S2), digested by restriction endonucleases (NotI/XbaI), and then cloned into the pMV261 backbone and target promoter plasmid DNA, and the regulator etbR was cloned into pMV261. Then reporter gene lacZ (amplified by PCR using two pairs of primers: 5’-TCTCAAGCTTATGAGGATGAGGGATCCGGAGCGATG-3’ and 5’-ATGCCGCTAGTTTATTGACCCAGACACCA-3’; Table S2) was cloned immediately downstream of target gene promoter DNA or etbR. To construct the pMZ+ plasmid, which was used as a positive control in our assays, the reporter gene lacZ was inserted into pMV261 downstream of the enhanced promoter of hsp60 using HindIII/NheI. To construct the negative control plasmid pMZ−, the promoter region of pMZ+ was removed by XbaI/EcoRI double-digest and blunt-end ligation. The recombinant plasmids were then transformed into *M. smegmatis* MC2 155 to obtain corresponding reporter strains. All strains were grown in 7H9-Tw-glycerol-Kan medium at 37 °C for 48 h. Some cell suspension was then incubated into 7H9-Tw-glycerol-Kan liquid medium. β-gal activity was measured as described previously (27).

**SPR analysis**

The interaction between EMB and EtbR was analyzed on a BIAcore 3000 instrument (GE Healthcare) according to the BIAcore Sensor Surface Handbook. The assays were performed at 25 °C. EtbR proteins were covalently coupled to a CM5 sensor chip using standard amine-coupling chemistry. Briefly, the carboxylated dextran matrix was activated by injecting a mixture of 0.05 M N-hydroxysuccinimide and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (1:1). EtbR protein (5 μM) was then injected, followed by 1 M ethanolamine-HCl (pH 8.5) to remove noncovalently bound ligand. A running buffer (10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 50 μM EDTA, and 0.005% BIAcore surfactant P20) was used in all binding experiments, and a pulse of 10 mM glycine-HCl (pH 2.0) was used for regeneration. Biosensor surfaces were coupled to the final resonance values of ~5600 RU. Different amounts (60–480 μg/ml) of EMB were then passed over the chip. GTP and INH

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**Synergistic action of isoniazid and ethambutol**
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were substituted as negative controls. To assess the binding of DNA with proteins, biotinylated inhA::3xHis probes were immobilized onto SA chips at densities of ~600 RU. EtbR, Rv0275c, and EtbR-EMB were diluted in dialysis buffer (20 mM Tris-HCl, pH 6.5, 100 mM NaCl) at concentrations of 0.5, 1, and 2 μM protein and injected at 10 μl/min for 5 min. INH was substituted as a negative control. The response was measured in RU. Several overlay plots were produced to depict the interactions using BIAevaluation version 3.1 software.

Author contributions—C. Z. and Z.-G. H. investigation; C. Z., Y. L., L. H., and M. Y. methodology; C. Z. and M. Y. writing-original draft; C. Z., M. Y., and Z.-G. H. writing-review and editing; M. Y. data curation; M. Y. and Z.-G. H. supervision; M. Y. and Z.-G. H. funding acquisition; Z.-G. H. project administration.

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