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Activation of the Pro-drug Ethionamide Is Regulated in Mycobacteria*

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Alain R. Baulard‡, Joanna C. Betts§, Jean Engohang-Ndong‡, Selwyn Quan§, Ruth A. McAdam§, Patrick J. Brennan¶, Camille Locht‡, and Gurdyal S. Besra‖**

From ‡INSERM U447, Institut de Biologie de Lille, Institut Pasteur de Lille, 59019 Lille, France, §Glaxo Wellcome, Research and Development, Stevenage SG1 2NY, England, the ¶Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523-1677, and the ‖Department of Microbiology & Immunology, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, England

The anti-tuberculosis drug ethionamide (ETH), which is a structural analog of isoniazid (INH), is known to strongly inhibit mycolic acid synthesis in *Mycobacterium tuberculosis*. Although several targets have been identified for INH, only speculative information is available concerning ETH. Mutations within the promoter and the coding region of enoyl-acyl carrier protein reductase (InhA) were found to confer resistance to both drugs, thus leading to the impression that INH and ETH may share a common mode of action. However, a notable distinction between the two drugs lies in the lack of cross-resistance in clinical isolates. This may be attributed in part to the fact that the pro-drug INH must be activated via KatG, and no activation step for ETH has yet been described. Here we report the identification of an activator for ETH. The ETH activator (*Rv3854c*), which we have termed EthA, was found to be homologous to various monooxygenases and induced ETH sensitivity when overexpressed in mycobacteria. Interestingly, the neighboring open reading frame (*Rv3855*), which was found homologous to transcriptional repressors of the *tetR* family, led to ETH resistance when overexpressed. In addition, chromosomal inactivation of this gene by transposition led to ETH hypersensitivity. These data strongly suggest that *Rv3855*, which we have termed EthR, regulates the production of EthA, which subsequently activates the pro-drug ETH. This study opens up new avenues of research relating to ETH activation in mycobacteria, possibly leading to an improved efficacy of ETH and to the generation of new anti-mycobacterial agents.

The treatment of tuberculosis involves extremely lengthy and specialized chemotherapy regimens (1). The molecular composition and structural features of the mycobacterial cell envelope are thought to confer low permeability and thereby a basal resistance to most hydrophilic drugs (2). As a consequence, the lack of potency and protracted duration of drug administration are a major cause of rampant mutational drug resistance of *Mycobacterium tuberculosis* (3). An essential step in developing novel therapies for the treatment of *M. tuberculosis* infections is to determine why multidrug-resistant strains of *M. tuberculosis* are resistant to many existing anti-mycobacterial agents. Possible mechanisms of resistance include: alteration of the target enzyme that has become resistant to antibiotics; increased expression of the gene encoding the target enzyme; mutations causing impermeability of the mycobacterial cell to the antibiotic; and/or alterations of an activation mechanism. Thus, the urgent need to develop new therapies for treating *M. tuberculosis* infections requires the definition of the failures of existing treatments and the discovery of new drug targets.

The bacterial cell wall has been an effective target for many drugs (4). Many anti-tuberculosis agents, including ethambutol, cycloserine, isoniazid (INH),[1] ethionamide (ETH), thiocarlide, and the thiosemicarbazones are known to inhibit cell wall biosynthesis. INH (see Fig. 1), which is one of the most efficient and the most widely used anti-tuberculosis drugs, has been the subject of intensive research during the past decade (5–7). Both *M. tuberculosis* and *Mycobacterium bovis* BCG are extremely susceptible to INH (minimum inhibitory concentrations (MIC), 0.02–0.2 μg/ml), and earlier evidence suggested that INH specifically inhibits the synthesis of mycolic acids in *M. tuberculosis* and *M. bovis* BCG (8–11). ETH (see Fig. 1), a structural analog of INH, is a useful second line anti-tuberculosis drug. The two drugs have almost identical effects in that both strongly inhibit the synthesis of mycolic acids (12, 13). Banerjee and colleagues (14) demonstrated that a single mutation in the *inhA* gene (NADH-specific, 2-*trans*-enoyl-acyl carrier protein reductase) confers resistance to INH and ETH, leading to the impression that the mode of action of both drugs is identical. In addition, mutations within *katG*, encoding a catalase-peroxidase led to the majority of INH-resistant isolates (6), demonstrating that INH is a pro-drug and that an activated metabolite is responsible for its mode of action (15, 16). However, the notable distinction between the actions of ETH and INH resides in the lack of cross-resistance (4, 17). The majority of

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** A Lister Institute-Jenner Research Fellow. To whom correspondence should be addressed. Tel.: 0191-222-5412; Fax: 0191-222-7736; E-mail: g.s.besra@newcastle.ac.uk.

[1] The abbreviations used are: INH, isoniazid; ETH, ethionamide; ETH^R, ETH-resistant; ETH^s, ETH-susceptible; FAME, fatty acid methyl ester; MAME, mycolic acid methyl ester; TLC, Thin layer chromatography; ORF, open reading frame; TBAH, tetrabutylammonium hydroxide; *hyg*, hygromycin resistance gene; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); MIC, minimum inhibitory concentration; BCG, bacillus of Calmette-Guérin.

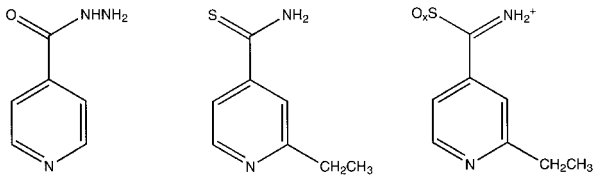

FIG. 1. **Structures of INH, ETH, and ETH *S*-Oxide.**

strains resistant to ETH are sensitive to INH, whereas some strains resistant to INH show slightly increased sensitivity to ETH (18). Thus, there are subtle discrepancies between biochemical and genetic information on the modes of sensitivity and resistance in the cases of INH and ETH.

Because INH requires activation via KatG, it is tempting to postulate an activation process for ETH. Inactivation of such an ETH-specific process could account for the lack of cross-resistance between the two drugs. Interestingly, ETH undergoes oxidation by rat liver microsomes to generate a highly reactive S-oxide, possibly a sulfinate (Fig. 1) that exhibits greater activity *in vitro* against *M. tuberculosis* than ETH itself (19–21). These observations, in addition to the fact that genetic alterations in *katG* do not confer resistance to ETH, have led us to the hypothesis that ETH needs to be activated through a KatG-independent mechanism.

In this report, we describe the cloning and characterization of the gene *Rv3855*, which we now term *ethR,* that confers resistance to ETH, but not to INH when it is overexpressed in either *Mycobacterium smegmatis*, *M. bovis* BCG, or *M. tuberculosis* on a multycopy vector. Furthermore, a transposon mutant of *ethR* leads to ETH hypersensitivity in *M. bovis* BCG. In addition, genetic and biochemical evidence suggests that *ethR* encodes a transcriptional regulator that is not directly implicated in mycolic acid biosynthesis but plays an important role in the regulation of a second open reading frame (ORF), which is responsible for the activation of ETH. Analysis of the locus surrounding *ethR* revealed the presence of an adjacent gene now termed *ethA*, which encodes a putative monooxygenase, the predicted activator of ETH. Overexpression of *ethA* led to hypersensitivity to ETH in mycobacteria. Thus, the data presented are compatible with the notion that EthR represses *ethA*, which encodes the equivalent protein of KatG implicated in the activation of ETH.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—M. smegmatis mc^{2}155, *M. bovis* BCG 1173P2 (WHO, Stockholm, Sweden), and *M. tuberculosis* H37Ra and their transformants were grown on 7H11 agar supplemented with oleic-albumin-dextrose-catalase enrichment (Difco, Detroit, MI) or on Sauton medium (22) supplemented with 0.001‰ $ZnSO_4$ and 0.25‰ Triton WR1339 (Sigma). *M. smegmatis* mc^{2}155 is an electroporation-efficient mutant of mc^{2}6 (23). *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* were transformed as described previously (24). Large scale cultures of mycobacteria were grown to mid-log phase (*M. bovis* BCG, 10–14 days; *M. smegmatis* mc^{2}155, 36 h; *M. tuberculosis* H37Ra, 12–16 days), harvested, washed with phosphate-buffered saline and stored at −20 °C until further use. *Escherichia coli* strains XL1-Blue (Stratagene, La Jolla, CA), NK5587, and their transformants were grown in Luria-Bertoni (LB) broth (Life Technologies, Inc.) and agar medium. *E. coli* SH305 was grown in 2YT (25) supplemented with 1% glucose. All strains were incubated at 37 °C. Antibiotics (Sigma) were added to media at the following concentrations: kanamycin at 20 μg/ml, tetracycline at 10 μg/ml, chloramphenicol at 100 μg/ml, and ampicillin at 100 μg/ml.

Plasmids and DNA Manipulation—The *E. coli*-mycobacterial shuttle vector pMV261 containing the *hsp60* promoter was used as described previously (26). Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals (Mannheim, Germany), and Vent DNA polymerase was purchased from New England BioLabs. All DNA manipulations were performed using standard protocols, as described by Sambrook *et al.* (25).

Identification of pETH80 and Transposon γδ Mutagenesis of pETH80 in E. coli—A genomic library of *M. tuberculosis* H37Rv, constructed by cloning 35- to 40-kb *Sau*3AI fragments of chromosomal DNA into the shuttle cosmid pYUB18 (27), was the source of cosmid pETH80 conferring ETH resistance to *M. smegmatis*. The minimal region of pETH80 involved in ETH resistance was identified by γδ transposition in *E. coli* as described by Guyer (28). Briefly, a suitable *E. coli* donor strain NK5587 (F′ *lacY*::Tn*9*) was transformed with pETH80. One isolated transformant was subsequently conjugated with *E. coli* SH305 (*recA*1 *srl*::Tn*10*) with selection for kanamycin-resistant/tetracycline-resistant exconjugants. Plasmid DNA was extracted from 50 double resistant exconjugants, retransformed into *M. smegmatis*, and scored for their ETH^R phenotype. Tn*1000* (γδ) insertions were localized by restriction mapping and sequencing using primers near the end of γδ (primer γδ1: 5′-CAACGAATTATCTCCTT-3′; primer γδ2: 5′-TCAATAAGTTATACCAT-3′).

Cloning and Expression of Rv3855 and Rv3854c—Rv3855 was cloned into the mycobacterial overexpression vector pMV261 as follows. PCR amplification was performed using the upstream primer 142: 5′-CCACCTCCGCGGCCAGTCAGG-3′ and the downstream primer 141: 5′-TTTGGCACTGAGAATTCACCGAGCACCC-3′, which contains an *Eco*RI restriction site (*underlined*). The 690-bp PCR product was digested with *Eco*RI and cloned into *Mlu*NI/*Eco*RI-restricted pMV261, giving rise to pMV261-*ethR*, where *ethR* is fused in-frame with the ATG initiation codon of *hsp60*. A similar strategy was used to construct the pMV261-based expression vector for *Rv3854c*. *Rv3854c* was amplified by PCR using the upstream primer 150: 5′-AGCACCTCGACGTTGTCATC-3′ and the downstream primer 149: 5′-ACGGATCCCCGCAAGAGCACCA-3′, which contains a *Bam*HI restriction site (*underlined*). The 1624-bp fragment was digested by *Bam*HI and cloned into *Mlu*NI/*Bam*HI-restricted pMV261, generating pMV261-*ethA*. The coding sequences of all amplified genes were verified by DNA sequencing after their cloning in pMV261.

Determination of the in Vivo Effects of ETH and INH on Fatty Acid and Mycolic Acid Synthesis in Mycobacteria—M. smegmatis mc^{2}155, *M. bovis* BCG, and *M. tuberculosis* were grown to mid-log phase (A_{600} ~0.3). ETH or INH were added at various concentrations followed by further incubation for 4 h (for *M. smegmatis*) or 24 h (for *M. bovis* BCG and *M. tuberculosis*). At this point, 1,2-[^{14}C]acetate (1 μCi/ml) (Amersham Pharmacia Biotech) was added and the cultures were further incubated with gentle agitation at 37 °C for 4 h (for *M. smegmatis*) or 24 h (for *M. bovis* BCG and *M. tuberculosis*). The resulting ^{14}C-labeled cells from ETH- or INH-treated cultures were harvested, washed twice with phosphate-buffered saline, resuspended into 3 ml of 15% tetrabutylammonium hydroxide (TBAH), and saponified at 100 °C for 15 h. After cooling, dichloromethane (4 ml), water (2 ml), and iodomethane (300 μl) were added, and the entire reaction mixture was agitated for 30 min. After centrifugation, the upper, aqueous phase was discarded and the lower, organic phase was washed twice with water, dried in a sand bath, and extracted twice with diethyl ether (3 ml) and the etheral extracts were dried and resuspended into 500 μl of dichloromethane for radioactivity counting. Equal volumes of this extract, which is composed of fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs), were separated by thin-layer chromatography (TLC) on silica gel (Merck 5735 Silica Gel 60 F_{254}, Darmstadt, Germany) and developed once in petroleum ether:acetone (95:5, v/v). Subsequent autoradiography revealed ^{14}C-labeled fatty acid and mycolic acid methyl esters after overnight exposure of the TLC plates to Kodak BioMax MR film.

Characterization of the M. bovis BCG Transposon Insertions—Genomic DNA (200–500 ng) of the mutagenized clones was digested with *Rsa*I (New England BioLabs). After heat inactivation of the restriction enzyme at 65 °C for 20 min, the digested DNA (40–100 ng) was ligated with T4 DNA ligase (New England BioLabs) at room temperature. PCR reactions were carried out in a total volume of 25 μl containing Amplitaq PCR buffer (Perkin-Elmer), 10% (v/v) dimethyl sulfoxide, 0.25 μM dNTPs, 0.4 μM 84L-F (5′-GTCATCCGGCTCATCACCAG-3′), and 0.4 μM 84L-R (5′-AACTGGCGCAGTTCCTCTGG-3′) primers, 4–10 ng of template DNA, and 0.5 unit of Amplitaq Gold (Perkin-Elmer). Thermal cycling was performed in a Perkin-Elmer 9600 machine with an initial denaturation at 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s, and a final extension of 72 °C for 7 min.

RESULTS

Identification of Cosmids Conferring ETH Resistance in M. smegmatis—The chromosomal region involved in ETH resist-

ance was sought using drug resistance imparted via gene overexpression by a plasmid vector, as a selection tool. A genomic library of DNA from *M. tuberculosis* H37Rv was screened using the ETH^S (MIC of 15 μg/ml) *M. smegmatis* host strain mc^2155 for clones that exhibited an ETH^R phenotype. Electrotransformants were plated in duplicate on medium containing kanamycin with ETH at 80 μg/ml. Colonies were scored as ETH^R if growth appeared on plates after 4 days of incubation at 37 °C. Single ETH^R colonies were re-streaked onto duplicate 7H10/oleic-albumin-dextrose-catalase plates containing kanamycin and INH (5 μg/ml). Specific ETH^R clones were selected that grew in the presence of ETH but remained fully sensitive to INH. The cosmids, which confer ETH^R, were recovered from the *M. smegmatis* transformants by electroduction into *E. coli* (29). Following analysis, all clones were found to contain the same cosmid (pETH80), which corresponded to a minimum of two chromosomal regions, probably associated during the packaging process (data not shown).

Identification by Transposon γδ (Tn1000) Mutagenesis of the Minimal Region of pETH80 Responsible for ETH Resistance—The minimal region required for ETH resistance in *M. smegmatis* was determined by transposon mutagenesis of pETH80. The method, adapted from Guyer *et al*. (28) is based on the transposition in *E. coli* of γδ during F-mediated conjugal mobilization. Briefly, the mutagenesis technique is based upon the observation that conjugal transmission of pBR322 by the conjugative plasmid F is dependent on, or at least completely correlated with, the transposition of γδ from F to pBR322 (28). The transposition of γδ has also proved efficient on plasmids other than pBR322 (30). The cosmid pETH80 identified in this study is an *E. coli*-mycobacterial shuttle vector based on ColE1 origin of replication. Thus, the mapping process of the minimal region of pETH80 responsible for ETH^R in mycobacteria was achieved in two steps. First, mutagenesis of pETH80 in *E. coli* before the re-introduction of the derivatized DNA into mycobacteria allowed the analysis of insertion mutations. Of the 50 clones analyzed, 38 γδ insertions were located in the inserted portion of pETH80. Three γδ insertions were able to abolish ETH^R in *M. smegmatis*. Restriction analysis of these clones revealed that these γδ insertions were located within 0.6 kb of DNA. Sequencing of the regions located left and right of γδ (with primers γδ1 and γδ2) revealed that all three insertions disrupted the same ORF annotated *Rv3855* in the *M. tuberculosis* H37Rv genome data base, which was thus termed *ethR*.

Cloning and Overexpression of ethR (Rv3855) in Mycobacteria—To avoid possible interference of other ORFs of pETH80 in association with ETH^R, the coding region of *ethR* was amplified by PCR and cloned in-frame with *hsp60* into pMV261. The resultant plasmid pMV261-*ethR* was transformed into *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis,* and the MICs of the transformed bacteria were compared with those of untransformed strains and with those of strains containing the original pETH80. The MICs were determined by plating serial dilutions onto medium containing kanamycin plus 0–200 μg/ml ETH in increments of 10 μg/ml or 0–10 μg/ml INH in increments of 1 μg/ml. The MIC was defined as the lowest concentration of ETH that inhibited the growth of 99% of the bacteria. Table I summarizes the results obtained, suggesting a direct correlation between the level of expression of *ethR* and ETH resistance. All three mycobacterial species transformed with pMV261-*ethR* remained sensitive to INH, thus demonstrating that overexpression of *ethR* specifically induces ETH resistance. In parallel, susceptibility assays with isoxyl, thiolactomycin, erythromycin, crystal violet, and streptomycin indicated that resistance was specific for ETH.

TABLE I
MICs (μg/ml) of ETH for mycobacteria harbouring ethR, ethA, or ethR KO

	pMV261	pETH80	pMV261-*ethR*	pMV261-*ethA*	*ethR*::*hyg*
M. smegmatis[a]	15	80	250	<1	NT[b]
M. bovis BCG[c]	2	25	35	NT	<0.6[d]
M. tuberculosis[e]	1	20	30	NT	NT

[a] Strain mc^2155.
[b] NT, not tested.
[c] Strain Paris 1173P2, except in *column* 5.
[d] Strain NCTC 5692.
[e] Strain H37Ra.

Selective Effects of ETH on Inhibition of Mycolic Acid Synthesis—Untransformed and pMV261-*ethR*-transformed *M. bovis* BCG were grown in the presence or absence of ETH at various concentrations, after which cultures were labeled with 1,2-[^{14}C]acetate. Combined MAMEs and FAMEs were extracted, resolved, and fractionated on TLC plates. Untransformed *M. bovis* BCG exhibited a clear decrease in the incorporation of radioactivity into MAMEs in the presence of ETH (from 0 to 100 μg/ml) (Fig. 2). Examination of the individual classes of mycolates revealed that the production of all species was specifically inhibited. In addition, the production of a yet to be described and identified product, possibly a mycolate-specific fatty acid precursor, was progressively inhibited after treatment with ETH (and with INH) (Fig. 3). The characterization of this product may be crucial for the identification of the specific target for ETH, but has been hampered by its relatively low abundance in the mycobacterial cell wall. In contrast, treatment of *M. bovis BCG* (pMV261-*ethR*) with ETH (0–100 μg/ml) had no dramatic effect on the synthesis of MAMEs and FAMEs (Fig. 2). Possible mechanisms related to ETH resistance may include detoxification of the drug or repression of an activation process of ETH leading to its active metabolite, conceivably an *S*-oxide type derivative (19). The latter seemed an attractive scenario, because EthR is homologous to bacterial regulatory proteins of the TetR family (see Fig. 4*B*). Many of the proteins of the TetR family are repressors. As for EthR (23.725 kDa), they all have similar molecular masses, from 21 to 25 kDa. The most conserved region between EthR and the TetR family members is characterized by a helix-turn-helix motif located in the N-terminal third of the protein. As illustrated in Fig. 4*A*, the signature pattern (PS01081) starts four residues before the helix-turn-helix motif and ends six residues after the motif.

Construction of an M. bovis BCG ethR Knock Out Mutant—Mutagenesis of *M. bovis* BCG NCTC 5692 was performed as described previously using the mycobacteriophage mini-transposon delivery system pJSC84 (31).[2] Individual mutants were isolated, and the transposon insertions were characterized by inverse PCR. A clone with a transposon insertion between nucleotides 4,327,971 and 4,327,972 in the *M. bovis* BCG genome was identified (Fig. 5). The insertion disrupted *ethR* between nucleotide positions 426 and 427, leading to the production of a truncated polypeptide of 142 amino acids (instead of 648 for the normal protein). The *M. bovis* BCG *ethR*::*hyg* strain was found to be extremely sensitive to ETH, with an MIC < 0.6 μg/ml (see Table I). The MIC for INH remained identical for *M. bovis BCG* and *M. bovis* BCG *ethR*::*hyg*, confirming that *ethR* was specific for ETH resistance.

Inhibition of Mycolic Acid Synthesis in M. bovis BCG (pMV261-ethR)—The isolated *M. bovis* BCG *ethR*::*hyg* clone was grown in the presence or absence of ETH and examined for the relative inhibition of mycolic acid synthesis. Concentrations as low as 2.5 μg/ml ETH completely abolished mycolic

[2] J. Cox, unpublished results.

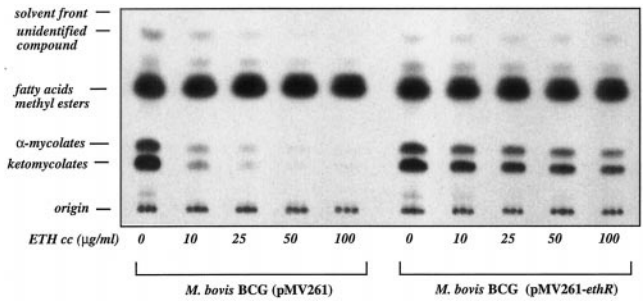

FIG. 2. **The dose-response effects of ETH on fatty acid and mycolic acid synthesis in *M. bovis* BCG (pMV261) and *M. bovis* BCG (pMV261-*ethR*).** The inhibitory effect on the incorporation of 1,2-[^{14}C]acetate was assayed by labeling in the presence of increasing concentrations of ETH and terminated by addition of 15% TBAH at 100 °C overnight. The corresponding FAMEs and MAMEs were isolated, spotted on aluminum-backed TLC plates, which were developed once in petroleum ether:acetone (95:5, v/v), and exposed overnight to a Kodak BioMax MR film.

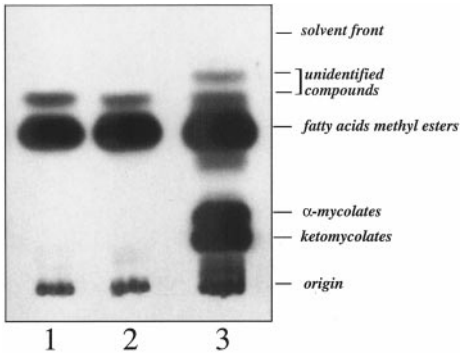

FIG. 3. **Effect of INH and ETH on fatty acid and mycolic acid synthesis.** The samples are FAMEs and MAMEs from *M. bovis* BCG + ETH 40 μg/ml (*lane 1*), *M. bovis* BCG + INH 10 μg/ml (*lane 2*), and *M. bovis* BCG (*lane 3*).

acid synthesis in comparison to 50 μg/ml for the wild-type *M. bovis* BCG strain (Fig. 6). This supported our hypothesis that *ethR* is involved in the repression of the formation of the active metabolite of ETH. Interestingly, in the absence of ETH, the FAME and MAME profiles of the mutant were unaffected, confirming the notion that *ethR* is not directly implicated in mycolic acid synthesis, but may rather be related to ETH activation.

Identification of the Gene Encoding the Putative ETH Activator—It is not unusual to find a gene under the control of a transcriptional regulator encoded by the neighboring ORF. The intergenic region located between *Rv3854c* and *Rv3855* (*ethR*) is 76 bp long, and the two genes are transcribed in opposite directions. This situation is consistent with the presence of divergent promoters in this region (32). For some regions of divergent transcription described so far, one transcript determines a regulatory molecule and the other determines a non-regulatory polypeptide. The regulatory molecule may act within the divergent transcript unit to control transcription of the non-regulatory polypeptides; often it also regulates its own synthesis (32). Interestingly, *Rv3854c*, the adjacent ORF to *ethR*, is homologous to various monooxygenases (Fig. 4*C*). Thus, *Rv3854c* could be a candidate to play a role equivalent to eukaryotic monooxygenases in the generation of active metabolites of ETH, notably *S*-oxides (20, 21). To test this hypothesis, *Rv3854c* was overexpressed in *M. smegmatis* (pMV261-*ethA*) and the recombinant strain was examined for ETH sensitivity. As anticipated, *M. smegmatis* (pMV261-*ethA*) was hypersensitive to ETH (Table I), and a dramatic inhibition of mycolic acid synthesis was observed at very low concentrations of ETH in comparison to wild-type *M. smegmatis* (Fig. 7). These results strongly suggest that *Rv3854c*, which we have now termed *ethA*, plays a role equivalent to *katG* for INH, but in relation to ETH.

DISCUSSION

INH and ETH are specific anti-tuberculosis drugs, which inhibit mycolic acid synthesis through InhA as suggested by resistance to both ETH and INH of an *inhA* mutant (14). Moreover, by the use of microarray hybridization, Wilson and coworkers (33) recently demonstrated that ETH treatment of *M. tuberculosis* induced the same genes that were induced by INH. However, Fattorini and coworkers (17) recently reported that of 46 INH-resistant strains of *M. tuberculosis* isolated from Italian patients only two were also ETH-resistant. Before INH exerts its lethal effect it must be converted to an active form, possibly an isonicotinic acyl anion (34) or an isonicotinic acyl radical (35) produced by the catalase-peroxidase KatG. ETH and other thioamides are sulfur-containing compounds, which are known to be substrates for oxidative catalysts, such as flavin-containing monooxygenases and cytochrome P-450 monooxygenases. An NADPH-dependent oxidation of ETH has previously been demonstrated in rat microsomes (20). More recently, Johnsson *et al*. (34) suggested that *in vitro* oxidation of ETH produces electrophilic intermediates (*S*-oxides) capable of undergoing addition reactions to nucleophilic protein side chains (35). During the 1950s ETH and, subsequently, prothionamide were introduced in the treatment of tuberculosis and were deemed clinically as effective as dapsone in the treatment of leprosy (36). However, 25% of the patients suffered from various gastrointestinal symptoms and sometimes jaundice, especially when ETH was combined with rifampicin (37). The hepatotoxicity induced by administration of ETH and thionicotinamide was decreased by preadministration of methimazole. Preadministration of methimazole was also shown to decrease the levels of excretion of thionicotinamide *S*-oxide, indicating that thioamide *S*-oxidation, mediated by the flavin-containing monooxygenases, may be linked to the initiation of hepatotoxicity induced by these thioamides (38).

Here we describe evidence that ETH is activated by the monooxygenase homolog *Rv3854c* (EthA). EthA would then be the equivalent of KatG for INH. Thus, in a similar fashion by which mutations in *katG* abolish INH sensitivity without leading to ETH resistance, we would expect that mutations in *ethA* lead to ETH resistance without affecting INH sensitivity. Studies are currently in progress examining clinical isolates for point mutations within *ethA*. Overexpression of *ethA* led to a dramatic increase in ETH sensitivity of *M. smegmatis* and clearly indicated that only a small proportion of ETH is activated when mycobacterial cells are grown under laboratory conditions. Alternatively, when administered to humans, ETH may be activated by either eukaryotic oxidative processes as mentioned earlier or by EthA (or perhaps a combination of both). Thus, determining the respective contribution of the bacterial and the eukaryotic activation of thioamides to their ultimate necrogenic forms would be crucial to understand the impact of EthA on the efficacy of ETH *in vivo* and to help designing new improved versions of ETH.

ETH resistance may also be mediated by the overproduction of the putative repressor EthR. As a regulator, it is logical to assume that the production of EthR is also regulated, perhaps by signals external to the bacteria. Thus, any agent able to block EthR, or any physiological condition down-regulating *ethR* may favor the production of EthA and lead to the activation of substantial amounts of ETH, thereby increasing the sensitivity of the bacilli to ETH. Overexpression of *ethA* dramatically decreased the MIC of *M. smegmatis* for ETH to a level

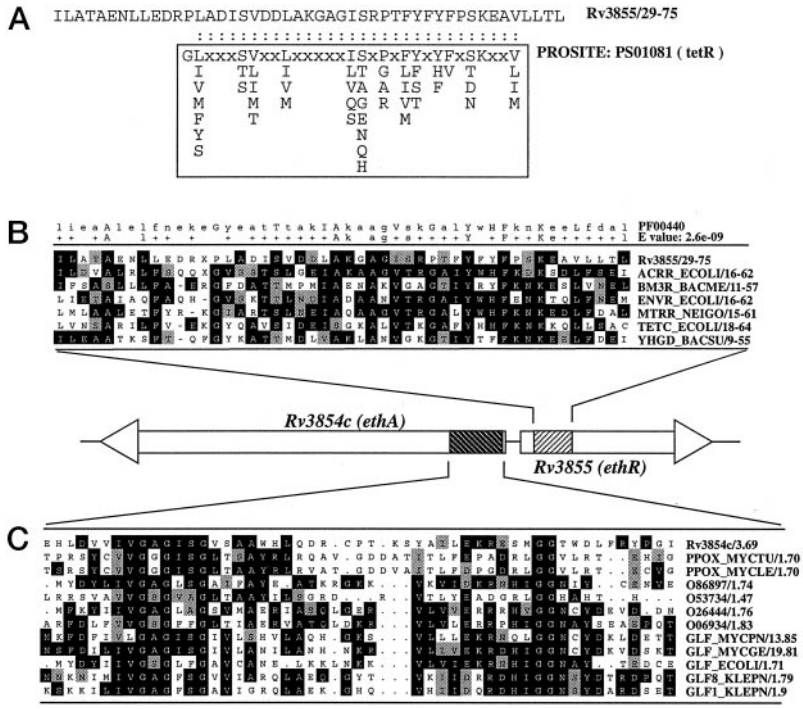

FIG. 4. **Locus *Rv3854c* and *Rv3855* of *M. tuberculosis* H37Rv.** *A*, the *tetR* family members are characterized by a helix-turn-helix motif located in the initial third of the protein. The signature pattern is a conserved domain, which starts four residues before the helix-turn-helix motif and ends six residues after the motif. With the exception of Pro[41], the *Rv3855* (*ethR*) region from Pro[41] to Val[71], fits the "Prosite" signature PDOC00830 (*tetR*), strongly suggesting that *Rv3855* (*ethR*) is a transcriptional regulator. *B*, the *tetR* consensus as determined at the Sanger center is named PF00440. A few members of the family have been chosen, and their PF00440 domain were aligned with *Rv3855* (*ethR*). A more detailed and exhaustive alignment is available from Sanger on the Web. *C*, *Rv3854c* (*ethA*) belongs to a large and diverse family of monooxygenases. The region homologous to monooxygenases is located in the N-terminal part of the protein.

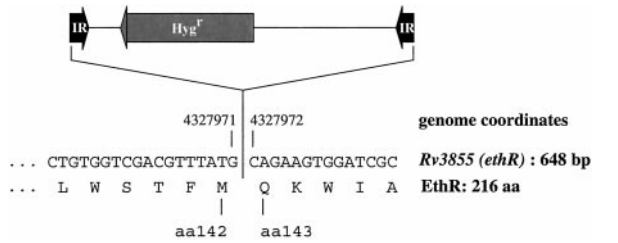

FIG. 5. **Positioning of the transposon insertion in the genome of the clone *M. bovis* BCG NCTC 5692 *ethR::hyg*.**

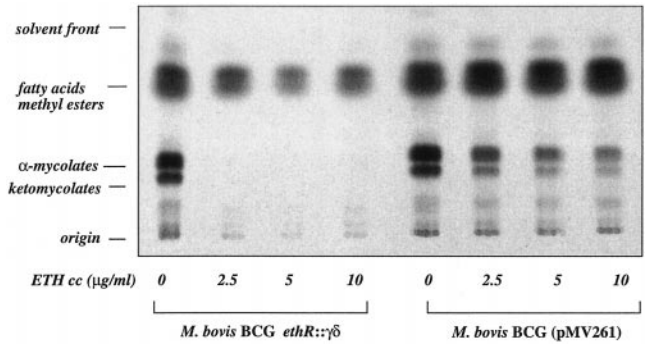

FIG. 6. **The dose-response effects of ETH on FAME and MAME profiles in *M. bovis* BCG NTC 5692 and *M. bovis* BCG NTC 5692 *ethR::hyg*.**

comparable to the MIC of *M. tuberculosis* (see Table I). This suggests that in part the innate relative resistance to ETH of *M. smegmatis* is associated with a low production of EthA and thus, indirectly, with EthR. Perhaps this mechanism may also account for the various susceptibility profiles to ETH displayed by other mycobacterial species.

Interestingly, although synthesis of α and α' mycolates is insensitive to ETH in *M. smegmatis* overexpressing *ethR,* synthesis of epoxymycolates remained highly sensitive to the drug in this strain (data not shown). Thus, possible additional targets to InhA exist in relation to ETH and epoxymycolate synthesis, which has been previously shown to be highly sensitive to ETH (13).

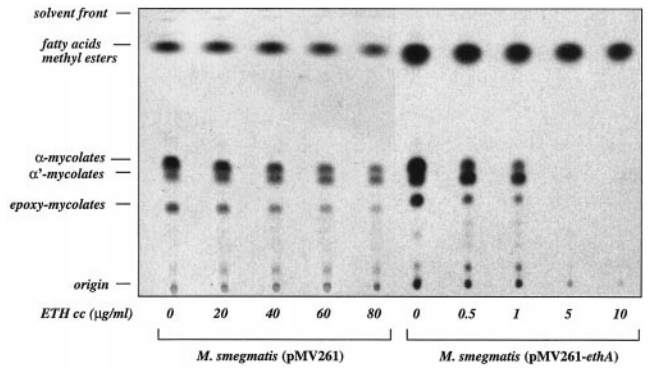

FIG. 7. **The dose-response effects of ETH on FAME and MAME profiles in *M. smegmatis* (pMV261) and *M. smegmatis* (pMV261-*ethA*).**

It is interesting to note that EthA and EthR orthologs are also present in *M. avium* and *M. leprae*. The *EthAR* loci of *M. avium* and *M. tuberculosis* share a very similar architecture. In both cases, the two genes are oriented "head to head" and are separated by a putative 76-bp divergent promoter. In contrast, the two *M. leprae* orthologs are not located in the same locus. The initiation codons of the *M. leprae EthA* and *EthR* are found in the genome in positions 86,698 and 1,235,214, respectively. The genome of *M. leprae* seems to be reduced to a minimal pool of genes essential to the survival of this strictly intracellular pathogen. In contrast to many genes of *M. leprae*, *EthA* and *EthR* are apparently not truncated and are highly similar to their orthologs in *M. tuberculosis* and *M. smegmatis*. Altogether, these observations suggest that these genes have an important role *in vivo*. In addition, the *M. tuberculosis* genome possibly encodes more than 30 monooxygenases, and the relative role of each enzyme in the oxidation of ETH remains to be assessed *in vivo*.

The understanding of the mode of action of ETH is valuable for the design of improved versions of the drug, for the elaboration of potentiating agents, and for the understanding of drug resistance. Finally, an intriguing question, which awaits further experimentation, concerns the possibility of an equivalent mechanism of regulation of *katG* and INH resistance.

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Activation of the Pro-drug Ethionamide Is Regulated in Mycobacteria

Alain R. Baulard, Joanna C. Betts, Jean Engohang-Ndong, Selwyn Quan, Ruth A. McAdam, Patrick J. Brennan, Camille Locht and Gurdyal S. Besra

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