Mycothiol biosynthesis is essential for ethionamide susceptibility in Mycobacterium tuberculosis

Catherine Vilchèze,1 Yossef Av-Gay,2 Rodgoun Attarian,2 Zhen Liu,3 Manzour H. Hazbón,4† Roberto Colangeli,3 Bing Chen,1 Weijun Liu,1 David Alland,3 James C. Sacchettini3 and William R. Jacobs Jr 1*

1 Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA.
2 Division of Infectious Diseases, University of British Columbia, Vancouver, BC V5Z 3J5, Canada.
3 Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843, USA.
4 Division of Infectious Disease, Department of Medicine and the Ruy V. Lourenço Center for the Study of Emerging and Re-emerging Pathogens, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA.

Summary

Spontaneous mutants of Mycobacterium tuberculosis that were resistant to the anti-tuberculosis drugs ethionamide and isoniazid were isolated and found to map to mshA, a gene encoding the first enzyme involved in the biosynthesis of mycothiol, a major low-molecular-weight thiol in M. tuberculosis. Seven independent missense or frameshift mutations within mshA were identified and characterized. Precise null deletion mutations of the mshA gene were generated by specialized transduction in three different strains of M. tuberculosis. The mshA deletion mutants were defective in mycothiol biosynthesis, were only ethionamide-resistant and required catalase to grow. Biochemical studies suggested that the mechanism of ethionamide resistance in mshA mutants was likely due to a defect in ethionamide activation. In vivo, a mycothiol-deficient strain grew normally in immunodeficient mice, but was slightly defective for growth in immunocompetent mice. Mutations in mshA demonstrate the non-essentiality of mycothiol for growth in vitro and in vivo, and provide a novel mechanism of ethionamide resistance in M. tuberculosis.

Introduction

The increase in drug resistance in Mycobacterium tuberculosis clinical isolates has impeded the full success of tuberculosis (TB) control. The WHO estimates that 4.3% of the newly and previously treated TB cases are multi-drug-resistant (MDR) meaning that these strains are resistant to at least the two best anti-TB drugs: isoniazid (INH) and rifampicin (Zignol et al., 2006). Alarming, there has been an emergence of M. tuberculosis strains resistant to four to seven TB drugs (termed XDR-TB) for extensively drug-resistant TB that have been associated with the rapid death of HIV-infected individuals (Gandhi et al., 2006; Shah et al., 2007). A more effective treatment for both MDR- and XDR-TB strains requires rapid detection and therefore understanding of all the mechanisms leading to drug resistance.

Isoniazid, the cornerstone of front-line TB treatment, shares a common target with the second-line TB drug ethionamide (ETH). Both INH and ETH are pro-drugs that require activation to form adducts with NAD to subsequently inhibit InhA, the NADH-dependent enoyl-ACP reductase (Dessen et al., 1995; Quemard et al., 1995) of the fatty acid biosynthesis type II system (Marrakchi et al., 2000). However, activation of INH and of ETH occurs through different pathways. INH is activated by the katG-encoded catalase peroxidase (Zhang et al., 1992; Wilming and Johnsson, 1999) to form the INH-NAD adduct (Rozwarski et al., 1998). ETH, on the other hand, is activated by the ethA-encoded mono-oxygenase (Baulard et al., 2000; DeBarber et al., 2000; Vannelli et al., 2002) to yield the ETH-NAD adduct (Wang et al., 2007). Mutations in either activator confer resistance to INH or ETH respectively (Piatek et al., 2000; Morlock et al., 2003; Ramaswamy et al., 2003; Hazbon et al., 2006). Co-resistance to INH and ETH can be mediated by mutations that alter the InhA target so as to prevent the INH-NAD or the ETH-NAD adduct from binding (Vilcheze et al., 2006), by mutations that cause InhA overexpression (Larsen et al., 2002; Vilcheze et al., 2006) or by mutations...
Table 1. *M. tuberculosis* strains used in this study.

| Strain            | Genotype         | Nucleotide | Amino acid        | Mutant generation                                      | Source                |
|-------------------|------------------|------------|-------------------|--------------------------------------------------------|-----------------------|
| H37Rv             | mshA1            | –          | –                 | Spontaneous mutant of H37Rv                           | Trudeau Institute     |
| mc<sup>4931</sup> | mshA1            | c382t      | Stop codon AA128  | Spontaneous mutant of H37Rv                           | This work             |
| mc<sup>4932</sup> | mshA4            | c817t      | R273C             | Spontaneous mutant of H37Rv                           | This work             |
| mc<sup>4933</sup> | mshA5            | g959t      | G299C             | Spontaneous mutant of H37Rv                           | This work             |
| mc<sup>4934</sup> | mshA6            | c991t      | Stop codon AA331  | Spontaneous mutant of H37Rv                           | This work             |
| mc<sup>4935</sup> | mshA7            | g1067a     | G356D             | Spontaneous mutant of H37Rv                           | This work             |
| mc<sup>4936</sup> | mshA8            | a1082c     | E361A             | Spontaneous mutant of H37Rv                           | This work             |
| mc<sup>4937</sup> | mshA9            | a1242del   | Frameshift        | Specialized transduction with phAE222                 | This work             |
| CDC1551           | mshA1            | –          | –                 | Specialized transduction with phAE222                 | CSU                   |
| mc<sup>4939</sup> | mshA10           | ∆mshA      |                   |                                                       |                       |
| Erdman            | mshA2            | a332g      | N111S             |                                                       | Trudeau Institute     |
| mc<sup>4942</sup> | mshA10           | ∆mshA      |                   |                                                       |                       |

CSU, Colorado State University.

in *ndh* that increase the intracellular NADH concentration, thereby competitively inhibiting the binding of the INH-NAD and ETH-NAD adducts to InhA (Miesel et al., 1998; Vilcheze et al., 2005). While the majority of clinical isolates resistant to INH or ETH have been shown to map to the activator genes (*katG*, *ethA*) or the *inhA* target, current studies still show that up to 22% of the INH-resistant *M. tuberculosis* clinical isolates have no mutations in the genes known to be involved in INH or ETH resistance (Habzon et al., 2006). In this study, to identify novel mutations conferring INH and ETH resistance, we isolated spontaneous mutants of *M. tuberculosis* in vitro and found that they map to *mshA*, a gene encoding a glycosyltransferase involved in mycothiol biosynthesis, suggesting that *mshA* was non-essential. Additional genetic and biochemical studies demonstrated that mycothiol biosynthesis is required for ETH susceptibility in *M. tuberculosis*. Furthermore, *in vivo* studies showed that mycothiol is not required for growth in mice.

**Results and discussion**

**Spontaneous mutants of *M. tuberculosis*, co-resistant to INH and ETH, map to mshA**

Numerous studies have demonstrated that there exist strains of *M. tuberculosis* that are resistant to INH and do not have mutations in the genes associated with INH resistance (*katG*, *inhA* structural gene and promoter, *ndh*) (Telenti et al., 1997; Piatak et al., 2000; Ramaswamy et al., 2003; Cardoso et al., 2004; Hazbon et al., 2006). To eliminate the majority of spontaneous mutants of *M. tuberculosis* that are singly resistant to INH and map to *katG*, we chose to isolate mutants that were co-resistant to INH and its structural analogue ETH. Samples of three independent *M. tuberculosis* H37Rv cultures were plated on media containing low concentrations of both INH and ETH [≤ 4-fold the minimum inhibitory concentration (MIC)]. Seven mutants were isolated at low frequencies (1–4 × 10<sup>−4</sup>). DNA sequence analysis of targeted genes in these seven strains revealed the absence of mutations in the genes known to mediate co-resistance to INH and ETH, namely *inhA* (the gene or its promoter region) and *ndh*. This analysis provided the evidence that these strains possessed mutations that conferred INH and ETH resistance and had not been previously identified in *M. tuberculosis*. The mutants were transformed with a cosmid genomic library of the drug-susceptible *M. tuberculosis* parent. The frequency of transformation was extremely low for most of the mutants (less than 100 transformants per transformation), and only one mutant, mc<sup>4936</sup>, which had the lowest level of INH resistance, yielded more than 1000 transformants. The cosmid transformants were screened for restoration of INH and ETH susceptibility. One potential complementing cosmid was isolated, sequenced and shown to contain the *mshA* gene, a gene characterized as mediating the first step in the biosynthesis of mycothiol (Newton et al., 2003; 2006), a key thiol in the family of Actinomycetes bacteria (Newton et al., 1996). A link between mycothiol biosynthesis and resistance to INH and ETH had been previously established in *Mycobacterium smegmatis* when transposon mutants in *mshA* were found to be resistant to INH (more than 25-fold) and ETH (sixfold) (Newton et al., 1999; 2003; Rawat et al., 2003). Subsequent sequence analysis of mc<sup>4936</sup> and the other mutants showed that all the *M. tuberculosis* H37Rv mutants had missense, nonsense or frameshift mutations in *mshA* (Table 1). The *mshA* mutants had various levels of resistance to INH (2- to 16-fold) and ETH (four- to eightfold) (Table 2). This is the first report that *mshA* mutations confer co-resistance to INH and ETH in *M. tuberculosis*.  

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The mshA mutants of M. tuberculosis are defective in the synthesis of mycothiol

The glycosyltransferase MshA is the first step in mycothiol biosynthesis that leads to the formation of N-acetyl-glucosamine inositol (Newton and Fahey, 2002; Newton et al., 2006). The biosynthesis of mycothiol requires five enzymes to form N-acetyl-cysteine glucosamine inositol or mycothiol from inositol-1-phosphate and UDP-N-acetyl-glucosamine: the glycosyltransferase MshA, the phosphatase MshA2, the deacetylase MshB, the cysteine ligase MshC and the acetyltransferase MshD (Fig. 1). To analyse the effects of the diverse mutations in mshA on the biosynthesis of mycothiol, the levels of mycothiol were measured in all the mutants using fluorescent high-performance liquid chromatography (HPLC) assay (Newton et al., 2000a). We found a dramatic reduction (83% to undetectable levels) in the concentration of mycothiol compared with wild type (Fig. 2A). As a control, we also measured the mycothiol level in an INH- and ETH-resistant M. tuberculosis inhA mutant, mc²4911 (Vilcheze et al., 2006), and found that this mutant had a concentration of mycothiol similar to that in wild type. Complementation of the mutants with pMV361::mshA, an integrative plasmid containing only the mshA gene of M. tuberculosis driven by the hsp60 promoter, restored mycothiol biosynthesis in all the mutants (Fig. 2B). This confirms that the defect in mycothiol biosynthesis was due to the mutations in mshA. Although mycothiol has been suggested to be essential for the growth of M. tuberculosis (Sareen et al., 2003), our data show that M. tuberculosis strains that do not produce mycothiol are viable.

Comparison of the MshA structures of M. tuberculosis and Corynebacterium glutamicum establishes a rationale for the inactivation of MshA in the mutants

Given the sequence identity (45.9%) between M. tuberculosis MshA and Corynebacterium glutamicum MshA (CgMshA) whose structure was recently determined (Vetting et al., 2008), the monomeric homology model of

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Table 2. INH and ETH minimum inhibitory concentrations (MICs).

| Strain      | MIC (mg l⁻¹) | MIC (mg l⁻¹) |
|-------------|--------------|--------------|
|             |   INH  ETH   | pMV361::mshA |
| H37Rv       | 0.06  2.5    | 0.06  2.5    |
| mc²4931     | 0.6   20     | 0.06  2.5    |
| mc²4932     | 0.4   10     | 0.06  2.5    |
| mc²4933     | 0.6   20     | 0.12  2.5    |
| mc²4934     | 1     10     | 0.5   2.5    |
| mc²4935     | 1     10     | 0.06  2.5    |
| mc²4936     | 0.12  20     | 0.06  2.5   |
| mc²4937     | 0.5   20     | 0.12  2.5    |
| mc²4938     | 0.06  >20    | 0.06  2.5    |
| CDC1551     | 0.06  2.5    |             |
| mc²4939     | 0.12  >20    | 0.06  2.5    |
| Erdman      | 0.06  2.5    |             |
| mc²4942     | 0.06  15     | 0.06  2.5    |

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Fig. 1. Mycothiol biosynthesis pathway. The first step in the biosynthesis of mycothiol (MSH) is the coupling of UDP-N-acetyl glucosamine and inositol-1-phosphate followed by dephosphorylation, carried out by the glycosyltransferase MshA and the phosphatase MshA2 respectively. The deacetylase MshB removes the acetyl group on the glucosamine which allows for the addition of a cysteine group on the free amino group by the ligase MshC. The last step is the acetylation of the cysteine amino group by the acetyltransferase MshD.
M. tuberculosis MshA was created using CPHmodels 2.0 with the UDP-complexed CgMshA (PDB code 3C4Q) as template (Lund et al., 2002). Superimposition of the model of M. tuberculosis MshA (consisting of Arg46–Ile445) and the chain B from UDP/inositol-phosphate-bound CgMshA (PDB code 3C4V) yields an RMSD of 0.65 Å, indicating a high homology between each other (Fig. 3A).

Four of the mshA mutants have single amino acid mutation (Table 1). These four amino acids (Arg273, Gly299, Gly356 and Glu361) are conserved in CgMshA (as Arg231, Gly263, Gly319 and Glu324) (Fig. 3C). Each of these amino acids plays an important role in either the substrate binding or the domain interaction (Fig. 3B). The side-chain amines of Arg273 interact with the β-phosphate of UDP via hydrogen bonding. This arginine is also one of the major determinants of the orientation of the inositol-phosphate as its side-chain lies against the face of inositol. Gly299 is not in the vicinity of the active site, but should be important for the protein stability as the next residue, Gly300, forms the only interdomain hydrogen bond with Gly61 (Fig. 3A). Although not directly seen in the model, Gly356 was proposed to be involved in the binding of the N-acetyl-glucosamine moiety which shall be transferred from UDP to inositol (Vetting et al., 2008). In mc²⁴⁹³⁶, the Glu361Ala mutation removes the side-chain carboxylate that forms hydrogen bonds with the 2′- and 3′-hydroxyls from the ribose moiety of UDP, which could result in the inactivation of MshA.

The other mshA mutants had either nonsense or frameshift mutations (Fig. 3C). In mc²⁴⁹³¹ and mc²⁴⁹³⁴, the nonsense mutations caused the loss of active-site elements. In mc²⁴⁹³⁷, the truncation of the protein was close to the C-terminus and the active site was unlikely to be affected. Herein the inactivation of MshA could be explained by the protein’s characteristic folding. Based on the homology model of MshA, each monomer is composed of N-terminal and C-terminal domains. Towards the end of C-terminus, a large α-helix spanning Cys409 to Ile445 crosses back to the N-terminal, which is likely to stabilize the overall folding of the protein. Therefore the mutations within this α-helix, such as in mc²⁴⁹³⁷, would detrimentally affect the conformation of MshA leading to its inactivation.

* = no detectable level

**Fig. 2.** Mycothiol contents in M. tuberculosis mshA mutants and complemented strains. The strains were grown to stationary phase. Mycothiol contents were measured in triplicate for the M. tuberculosis mshA point mutants (A) and their complemented strains (B) as well as for the mshA null mutants (C) and their complemented strains (D) as described in Experimental procedures. c = pMV361::mshA.
Co-resistance to INH and ETH is not mediated by \textit{inhA} overexpression nor by increased NADH/NAD\textsuperscript{+} ratios in \textit{mshA} mutants of \textit{M. tuberculosis}.

Although co-resistance to INH and ETH had been previously identified in \textit{M. smegmatis} \textit{mshA} transposon mutants (Rawat et al., 2003), no mechanism of resistance had been identified to link mycothiol biosynthesis with INH and ETH resistance (Koledin et al., 2002; Rawat et al., 2003). Previously, co-resistance to INH and ETH has been shown to be conferred by three different mechanisms: (i) structural mutations in the \textit{inhA} target (Banerjee et al.,...
Identification of a new ethionamide resistance mechanism

Isolation of precise null deletions of mshA in various M. tuberculosis strains

Introduction of a wild-type copy of mshA restored ETH sensitivity (Table 2) and mycothiol content (Fig. 2B) in all the mshA mutants, but a subset of the mutants (3/7) did not regain INH susceptibility (Table 2). We reasoned that these strains must have acquired secondary mutations to compensate for the loss of mycothiol and that these mutations were also mediating the INH resistance. Although further studies will be needed to identify such mutations, we could hypothesize from these complementation studies that the loss of the mshA gene would mostly confer ETH resistance. To test this possibility, precise null mutants of mshA were generated in three reference strains of M. tuberculosis using specialized transduction (Bardarov et al., 2002) (Fig. S1, Table 1). The H37Rv, CDC1551 and the Erdman ΔmshA strains were resistant to ETH, but only the CDC1551 ΔmshA strain showed a twofold increase in INH MIC (Table 2). Previously, deletions in mshB (Buchmeier et al., 2003) and mshD (Buchmeier et al., 2006) had been isolated in M. tuberculosis, and only the mshB mutant was shown to be INH-resistant. Our data show that the main drug resistance phenotype of the M. tuberculosis mshA null mutant is resistance to ETH.

All three M. tuberculosis mshA deletion mutants failed to produce any detectable level of mycothiol (Fig. 2C). Complementation of the null mutants with pMV361::mshA restored ETH susceptibility (Table 2) and mycothiol production (Fig. 2D). To rule out the possibility that the lack of mycothiol might cause a compensatory phenotype, we measured total thiol concentrations in the cells and found that the null mutants’ thiol concentrations were reduced by 53%, 77% and 82% in M. tuberculosis H37Rv, CDC1551 and Erdman respectively.

A previous study had suggested that the mshA gene was essential in M. tuberculosis (Buchmeier and Fahey, 2006). The discovery of frameshift mutations within the
mshA gene, followed by our successful construction of null mshA deletions in three independent M. tuberculosis strains, demonstrates that mshA is not an essential gene in M. tuberculosis. Although both studies attempted to generate null mshA mutants in M. tuberculosis using specialized transduction, it would be difficult to extrapolate why one was successful and the other one was not. We can only note that the M. tuberculosis mshA deletion strains were obtained after a very long incubation at 37°C (8 weeks). Furthermore, this study also confirms that mycothiol is the major thiol in M. tuberculosis as it represents more than 50% of the total thiol concentration in M. tuberculosis.

The mshA mutants require catalase to grow

As mycothiol has been suggested to be essential for the growth of M. tuberculosis (Sareen et al., 2003), we tested whether the mshA mutants had any growth defect in vitro.

![Graph](image)

**Fig. 5.** Growth of M. tuberculosis mshA mutants in vitro. A. Growth in liquid media (Middlebrook 7H9 supplemented with 0.2% glycerol, OADC and 0.05% tyloxapol) of four mshA point mutants (left) or two ΔmshA mutants and their complemented strains (right). B. Growth on solid media of H37Rv ΔmshA (top right), H37Rv ΔmshA pMV361::mshA (top left), CDC1551 ΔmshA (bottom right) and CDC1551 ΔmshA pMV361::mshA (bottom left) on Middlebrook 7H10 plates supplemented with 0.2% glycerol and ADS, ADS + beef liver catalase, ADS + oleic acid or OADC.

c = pMV361::mshA.
electrophiles, alkylating agents, antibiotics and oxidants (Rawat et al., 2007), it is not surprising that M. tuberculosis mutants producing no mycothiol require catalase for protection against toxic reactive oxygen intermediates.

Mycolic acid biosynthesis is not inhibited by ETH treatment of mshA mutants

The death of the tubercle bacillus following treatment with INH correlates with inhibition of the biosynthesis of the long-chain α-alkyl β-hydroxy fatty acids (up to 90 carbons in length) called mycolic acids, which are a major constituent of the mycobacterial cell wall (Winder and Collins, 1970; Takayama et al., 1972). ETH, based on its similarity to INH, has also been predicted and shown to inhibit mycolic acid biosynthesis (Winder et al., 1971; Quemard et al., 1992; Baulard et al., 2000). As mycothiol is not known to be involved in the FASII pathway, the resistance mediated by mshA could suggest that the lethal event occurs in some redox function. If so, it may be possible that INH and ETH treatment of mshA mutants does not confer resistance to mycolic acid inhibition by ETH or INH. Fatty acids were extracted from the wild-type M. tuberculosis strains, the ΔmshA mutants and the ΔmshA-complemented strains following INH or ETH treatment, and derivatized to their methyl esters. Analysis by thin-layer chromatography (TLC) allowed for the separation between the short-chain fatty acid (up to 26 carbons in length) methyl esters (FAMEs) and the long-chain mycolic acid methyl esters (MAMEs) (Fig. 6). Treatment of the wild-type M. tuberculosis strains and the ΔmshA-complemented strains with INH or ETH resulted in inhibition of mycolic acid biosynthesis as shown by the absence of MAMEs on TLC. In contrast, the ΔmshA mutants were resistant to mycolic acid inhibition upon treatment with ETH, but not with INH (Fig. 6).

The mshA mutants support the premise that ETH inhibits mycolic acid biosynthesis as the mshA mutants were resistant to mycolic acid biosynthesis inhibition upon ETH treatment. Previous studies have shown four different mechanisms of ETH resistance in tubercle bacilli, including: (i) target modification (Banerjee et al., 1994; Vilcheze et al., 2006), (ii) target overexpression (Larsen et al., 2002), (iii) intracellular NADH/NAD+ ratio alteration (Miesel et al., 1998; Vilcheze et al., 2005) and (iv) ETH activator inactivation (Baulard et al., 2000; DeBarber et al., 2000). All four of these phenomena are consistent with ETH being a pro-drug that is activated to form an adduct with NAD and this ETH-NAD adduct inhibits InhA, which results in mycolic acid biosynthesis inhibition (Vilcheze and Jacobs, 2007b; Wang et al., 2007).

To address the mechanism by which mutations in mshA confer ETH resistance, we can now rule out a number of these known mechanisms. Quantitative PCR analysis demonstrated that the inhA mRNA was not upregulated thereby suggesting InhA was not overexpressed. Moreover, we measured NADH/NAD+ ratios and found no increase in NADH concentration in the mshA mutants. All of these data, coupled with the lack of resistance to INH and the high resistance to ETH, allow us to hypothesize that mycothiol plays a role either in the activation step of ETH or in the formation of the ETH-NAD adduct.

Mycothiol promotes ETH activation by the ethA-encoded mono-oxygenase

As the null mutants showed low (twofold the MIC) to no resistance to INH but showed a high level of resistance to ETH (≥6-fold the MIC), we therefore postulated that mycothiol could be involved in either ETH activation or ETH-NAD adduct formation in M. tuberculosis. ETH is activated by the NADPH-specific FAD-containing mono-oxygenase EthA (Baulard et al., 2000; DeBarber et al., 2000; Vannelli et al., 2002). We tested the NADPH-dependent mono-oxygenation of ETH by EthA in the presence of mycothiol, and observed an increase in the rate of reaction directly proportional to the increase in mycothiol concentration, suggesting that mycothiol plays a role in

Fig. 6. Fatty acid methyl ester (FAME) and mycolic acid methyl ester (MAME) analyses of ΔmshA mutants. M. tuberculosis wild-type strains, ΔmshA and complemented strains were treated with INH (0.5 mg l−1) or ETH (15 mg l−1) for 4 h, and then labelled with [1-14C]-acetate for 20 h. Fatty acids and mycolic acids were saponified, methylated, extracted and separated by thin-layer chromatography. 14C-labelled FAMEs and MAMEs were detected by autoradiography after 36 h exposure at −80°C. c = pMV361::mshA.

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Table 3. The effect of mycothiol on EthA activity.

| [Mycothiol] (μM) | [NADPH] s⁻¹ μM s⁻¹ | NADPH/EthA mmol s⁻¹ (mg protein)⁻¹ |
|------------------|----------------------|----------------------------------|
| 0                | 0.0071               | 1.24 × 10⁻⁴                      |
| 4.38             | 0.0074               | 1.29 × 10⁻⁴                      |
| 17.5             | 0.0094               | 1.64 × 10⁻⁴                      |
| 26.2             | 0.0111               | 1.94 × 10⁻⁴                      |
| 43.8             | 0.0127               | 2.23 × 10⁻⁴                      |

the activation steps rather than in the formation of the ETH-NAD adduct (Table 3). Furthermore, replacing mycothiol by a different thiol, such as reduced glutathione, had no effect on the oxidation rate of NADPH (data not shown). This suggests that the increase in EthA activity upon the addition of mycothiol is specific to mycothiol, and does not occur in the presence of another thiol. To test if mycothiol was also required for the formation of the ETH-NAD adduct, the rate of inhibition of InhA by ETH in the presence of NAD⁺, NADPH, EthA and mycothiol was also measured. No formation of the ETH-NAD adduct was observed in these conditions (data not shown), which suggests that mycothiol is not involved in the formation of the ETH-NAD adduct. The mycothiol-dependent increase in the rate of NADPH conversion during the activation of ETH by EthA suggests that mycothiol promotes the activation of ETH by EthA. Two other anti-TB drugs, isoxyl and thiacetazone, are also activated by EthA (Dover et al., 2007). We therefore tested if the mshA mutants (null and point mutants) were also resistant to isoxyl and thiacetazone and found that they were fully sensitive to both drugs (data not shown). This implies that mycothiol is solely involved in the activation of ETH. We could hypothesize that mycothiol either stabilizes the intermediates formed upon activation of ETH or forms a complex with the active form of ETH, which allows for the formation of the ETH-NAD adduct. More in-depth studies are necessary to fully understand which role mycothiol plays in the activation step.

Mycothiol is not required for M. tuberculosis growth in vivo

Mycothiol has been postulated to be essential for M. tuberculosis growth in vivo. The mshA mutant mc²4936, which does not synthesize mycothiol, was chosen to study the survival of immunocompetent C57Bl/6 mice and immunocompromised SCID mice following aerosol infection. No significant difference in survival was observed between mice infected with wild-type M. tuberculosis and the mycothiol-deficient mshA mutant (Fig. 7A). Interestingly, the SCID mice infected with the complemented strain (complementation was done with a replicative plasmid expressing mshA) survived 30 days longer than the parent strain. In vivo growth of the mshA mutant mc²4936 in the lungs of immunocompromised and immunocompetent mice was also measured. In SCID mice, the mshA mutant and the wild-type M. tuberculosis strains grew at the same rate, while the complemented strain grew slightly more slowly, which might explain the differences in survival rates (Fig. 7B). In C57Bl/6 mice, the mshA mutant growth was slightly defective after 3 weeks but at week 8 of infection the mycobacterial burden in the lung was comparable between the mutant and the wild-type strain (Fig. 7B).

Our demonstration that M. tuberculosis ΔmshA strain requires catalase to grow in vitro, but not in mice suggests that either the host is not an oxidatively stressed environment or growth in mice induces alternative thiols that may compensate for the absence of mycothiol. Further studies will be required to solve this paradox.

Concluding remarks

A novel mechanism of ETH resistance has been discovered in M. tuberculosis which demonstrates that mycothiol plays a role in the pro-drug activation by the ethA-encoded mono-oxygenase. As mono-oxygenases are known to mediate detoxifying reactions, it is reasonable to assume that mycothiol plays a role in other, as yet unidentified, detoxifying reactions. This resistance is a loss of function and is consistent with mycothiol playing a role in the ETH activation process. The requirement for mycothiol in the inhibition of mycolic acid biosynthesis by ETH supports the model that ETH, upon activation with EthA, forms an adduct with NAD, which subsequently inhibits InhA. We hypothesize that novel drugs that bypass this activation step and target InhA directly should be developed as they could lead to the killing of M. tuberculosis cells.

Interestingly, the first study by Dubos and Middlebrook (1947) of a medium to grow tubercle bacilli (now referred as Middlebrook 7H9 with OADC supplement) did not add beef liver catalase. A few years later, the discovery by Middlebrook that INH-resistant mutants of M. tuberculosis were catalase-negative (Middlebrook, 1954; Middlebrook et al., 1954) initiated the need to add catalase to the media to isolate and grow M. tuberculosis INH-resistant strains. Our study shows that there exists at least one class of INH- or ETH-resistant M. tuberculosis mutants that would not readily grow on media without catalase and may provide an explanation why certain clinical isolates are difficult to grow. Furthermore, the finding that three mshA mutants, when complemented with a wild-type copy of mshA, still possessed a low-level resistance to INH but were fully sensitive to ETH suggests that these mutants must have acquired a secondary mutation. Therefore, there may exist addi-
tional mechanisms of INH resistance yet to be discov-
ered involving other redox pathways.

Experimental procedures

Bacterial strains, plasmids, phages and media

The M. tuberculosis strains (H37Rv, CDC1551 and Erdman) were obtained from laboratory stocks. The strains were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% (v/v) OADC enrichment (Difco), 0.2% (v/v) glycerol and 0.05% (v/v) tyloxapol. The solid medium used was the same as described above, with the addition of 1.5% (w/v) agar. The plasmids pMV261 and pMV361 were obtained from laboratory stocks. Hygromycin was used at concentrations of 50 mg l\(^{-1}\) for mycobacteria and 150 mg l\(^{-1}\) for Escherichia coli. Kanamycin was used at concentrations of 20 mg l\(^{-1}\) for mycobacteria and 40 mg l\(^{-1}\) for E. coli.

Isolation of INH- and ETH-resistant spontaneous mutants

Mycobacterium tuberculosis H37Rv mutants were isolated from non-mutagenized cultures grown in the media described above. The cultures were incubated by shaking at 37°C to late log phase. Ten-fold serial dilutions were then plated on agar plates (media described above) containing INH (0.2 \(\mu\)g ml\(^{-1}\)) and ETH (5 or 10 \(\mu\)g ml\(^{-1}\)). The plates were then incubated at 37°C for 6 weeks.

MIC determination

The strains were grown to an OD\(_{600}\) = 1.0. Ten-fold serial dilutions were plated on plates containing INH (0, 0.1, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.8, 1 \(\mu\)g ml\(^{-1}\)) or ETH (0, 2.5, 5, 10, 15, 20 \(\mu\)g ml\(^{-1}\)). The MIC was determined as the concentration of drug that reduced the number of colony-forming units (cfu) ml\(^{-1}\) by 99%. MICs were also determined using the MTT assay (Martin et al., 2005).

Determination of NADH and NAD\(^+\) cellular concentrations

Mycobacterium tuberculosis strains were grown to log phase. The cultures (12 ml) were spun, and the cell pellets were re-suspended in 0.2 M HCl (1 ml, NAD\(^+\) extraction) or 0.2 M NaOH (1 ml, NADH extraction). After 10 min at 55°C, the suspensions were cooled to 0°C, and neutralized by adding 0.1 M NaOH (1 ml, NAD\(^-\) extraction) or 0.1 M HCl (1 ml, NADH extraction). After centrifugation, the supernatants were collected, filter-sterilized and frozen. The concentration of

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Fig. 7. In vivo growth of wild-type M. tuberculosis H37Rv, mc\(^{24936}\) and its complemented strain mc\(^{24936}\)-c following low-dose (~10\(^{2}\) cfu) aerosol infection.

A. Survival in immunocompetent C57Bl/6 mice or immunocompromised SCID mice.

B. Growth in the lungs of C57Bl/6 mice or SCID mice.

C = pMV261::mshA.
NAD\(^+\) (or NADH) was obtained by measuring spectrophotometrically the rate of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction by the yeast type II alcohol dehydrogenase in the presence of phenazine ethosulphate at 570 nm, which is proportional to the concentration hotometrically the rate of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction by the yeast type II alcohol dehydrogenase in the presence of phenazine ethosulphate at 570 nm, which is proportional to the concentration

**Quantification of the inhA expression levels**

Total RNA extractions, cDNA synthesis and quantitative PCR with molecular beacons were performed in triplicate, as described previously (Larsen et al., 2002). *inhA* levels were normalized to *sigA* expression.

**Quantification of mycothiol contents**

The *M. tuberculosis* strains (45 ml) were grown to stationary phase for 2 weeks. Samples (9 ml) were transferred into conical tubes and centrifuged. The cell pellets were re-suspended in either 0.5 ml of mBr reagent (20 mM HEPES pH 8 + 2 mM monobromobimane in acetonitrile/water 1/1, v/v) or 0.5 ml of NEM reagent (20 mM HEPES pH 8 + 5 mM N-ethylmaleimide in acetonitrile/water 1/1, v/v)). The suspensions were heated at 60°C for 15 min and spun down. The supernatants (0.5 ml) were treated with 5 M methanethiol sulphonic acid (2 μl) and frozen. The samples were subjected to HPLC analysis as described earlier (Newton et al., 2000b).

**Quantification of the total thiol concentration**

*Mycobacterium tuberculosis* strains were grown to stationary phase and spun down. The cell pellets were washed with PBS and then re-suspended in 1 ml of PBS. Glass beads were added (0.2 ml), the suspensions were lysed using the Thermo Scientific FastPrep machine (45 s, speed 6, three times) and spun down, and the supernatants were filter-sterilized. The total thiol concentration was obtained using Ellman’s reagent by measuring spectrophotometrically, at 412 nm, a 1 ml solution containing 50 mM Tris (pH 8.0), 5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (10 μl), and the lysate to quantify (K\(_{\text{m}}\) \(=\) 2-nitro-5-thiobenzoate anion is 14150 M\(^{-1}\) cm\(^{-1}\)).

**Construction of the ΔmshA strains**

*Mycobacterium tuberculosis* mshA was replaced by a hygromycin cassette using the specialized transduction system previously described (Bardarov et al., 2002). Briefly, a 1 kb region flanking the left and right sides of mshA was PCR-amplified from *M. tuberculosis* genomic DNA using the following primers (the cloning sites are underlined):

- **LL** TTTTTTTTCTTACAAGATTGGGGCGCGCTGCAGCTCAC TG,
- **LR** TTTTTTTTCTATTCTGGGACGGCGCTGGGCGATCA AC,
- **RL** TTTTTTTTCTATTCTGGGACGGCGCTGGGCGATCA GC,
- **RR** TTTTTTTTCTATTCTGGGACGGCGCTGGGCGATCA TTG.

The PCR fragments were cut with Van911 and cloned into p004S. The resulting cosmids was sequenced before digesting with Pacl. The linearized cosmids was ligated to the Pacl-cut shuttle phasmid pAE159 and the resulting plasmid, pAE222, was packaged in vitro (GigaPack II, Stratagene). High-titre phage lysates were used to transduce *M. tuberculosis* H37Rv, CDC1551 and Erdman as described previously (Vilcheze et al., 2006). The plates were incubated at 37°C for 8 weeks. The transductants were checked for the deletion of mshA by Southern analysis (the genomic DNA of the transductants was cut with BglIII and probed with the right flank of mshA) (Fig. S1).

**Complementation of the mshA mutants**

The wild-type *M. tuberculosis* mshA gene was amplified from *M. tuberculosis* chromosomal DNA using the following primers: mshAF CGGCAGCTTTCCGTTCTGCAAGGAT GG (PvuII site underlined), mshAR GCGGAATTCTCGCG AAGGAGAAGTCAG (EcoRI site underlined). The PCR product was digested with PvuII and EcoRI and ligated to the replicative *E. coli* mycobacterial shuttle vector pMV261 (Stover et al., 1991) (http://www.aecom.yu.edu/tbresearch/Resources/Vectors/pMV261.html) restricted by PvuII and EcoRI or the integrative *E. coli* mycobacterial shuttle vector pMV361 (http://www.aecom.yu.edu/tbresearch/Resources/Vectors/ pMV361.html) (Stover et al., 1991) restricted by PvuII and EcoRI. The mshA mutant strains were then transformed with the plasmid pMV361::mshA or pMV261::mshA, using the following protocol. The strains (20 ml of cultures) were grown at 37°C to an OD\(_{600}\) = 0.8, washed twice with a 10% aqueous glycerol solution and re-suspended in 0.4 ml of a 10% aqueous glycerol solution. The cell suspensions (0.175 ml) were added to the plasmid (2 μl) and electroporated (2.5 kV, 25 μFd, 1000 Ω). Medium (1 ml) was added, and the suspension was incubated at 37°C for 24 h and plated on Middlebrook plates containing kanamycin (20 mg l\(^{-1}\)). The plates were incubated at 37°C for 6 weeks.

**Analysis of FAMEs and MAMEs**

*Mycobacterium tuberculosis* strains were grown to log phase, diluted to an OD\(_{600}\) = 0.3, treated with INH (0.5 mg l\(^{-1}\)) or ETH (15 mg l\(^{-1}\)) or no drug for 4 h, and then labelled with [\(^{14}\)C]-acacetate (10 μCi) for 20 h at 37°C. The cultures were spun down and washed once with water. The cell pellets were saponified, methylated, extracted and analysed by TLC using hexane/ethyl acetate 95/5 as the elution system (three elutions were performed) (Vilcheze and Jacobs, 2007a).

**EthA enzymatic activity assay**

The his-tagged EthA was produced, as previously described (Dover et al., 2007). The activity of EthA was determined by monitoring the absorbance decrease of NADPH at 340 nm (ε\(_{340}\) nm = 6.22 M\(^{-1}\) cm\(^{-1}\)). All the reactions were catalysed by ~1 μM EthA and performed in 50 mM Tris/ HCl, pH 7.5. Double reciprocal plots were used to determine the \(k_{\text{m}}\) of the oxidation of NADPH. For measuring the effect of mycothiol, reaction mixtures contained 200 μM NADPH and varying mycothiol concentrations.

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Mice experiments

SCID mice and C57Bl/6 mice (Jackson Laboratories) were infected via the aerosol route using a $10^{6}$ cfu ml$^{-1}$ mycobacterial suspension in PBS containing 0.05% Tween 80 and 0.04% antifoam. Three mice from each group were sacrificed after 24 h of infection and lung homogenates were plated on Middlebrook 7H10 plates containing the appropriate antibiotic to determine the initial infection dose. At 1, 3 and 8 weeks post infection, three mice were sacrificed to determine the bacterial burden in the lung, spleen and liver. Five mice were left for survival experiments.

Acknowledgements

Studies at Yossef Av-Gay laboratory are supported by the TB Veterans Charitable Foundation and the British Columbia Lung Association. We also acknowledge support for this work from NIH Grants AI43268 and AI46669, and from Grant 1P50GM6241 from the TB Structural Genomics Project. James C. Sacchettini acknowledges Robert A. Welch Foundation Grant A-0015.

References

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K.S., Wilson, T., et al. (1994) inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science 263: 227–230.

Bardarov, S., Bardarov, S., Jr, Pavelka, M.S., Jr, Sambandamurthy, V., Larsen, M., Tufariello, J., et al. (2002) Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in Mycobacterium tuberculosis, M. bovis BCG and M. smegmatis. Microbiology 148: 3007–3017.

Baulard, A.R., Betts, J.C., Erghoang-Ndong, J., Quan, S., McAdam, R.A., Brennan, P.J., et al. (2000) Activation of the pro-drug ethionamide is regulated in mycobacteria. J Biol Chem 275: 28326–28331.

Buchmeier, N., and Fahey, R.C. (2006) The mshA gene encoding the glycosyltransferase of mycothiol biosynthesis is essential in Mycobacterium tuberculosis. FEMS Microbiol Lett 264: 74–79.

Buchmeier, N.A., Newton, G.L., Koledin, T., and Fahey, R.C. (2003) Association of mycothiol with protection of Mycobacterium tuberculosis from toxic oxidants and antibiotics. Mol Microbiol 47: 1723–1732.

Buchmeier, N.A., Newton, G.L., and Fahey, R.C. (2006) A mycothiol synthase mutant of Mycobacterium tuberculosis has an altered thiol-disulfide content and limited tolerance to stress. J Bacteriol 188: 6245–6252.

Cardoso, R.F., Cooksey, R.C., Morlock, G.P., Barco, P., Cecon, L., Forestiero, F., et al. (2004) Screening and characterization of mutations in isoniazid-resistant Mycobacterium tuberculosis isolates obtained in Brazil. Antimicrob Agents Chemother 48: 3373–3381.

DeBarber, A.E., Mdluli, K., Bosman, M., Bekker, L.G., and Barry, C.E. (2000) Ethionamide activation and sensitivity in multidrug-resistant Mycobacterium tuberculosis. Proc Natl Acad Sci USA 97: 9677–9682.

Dessen, A., Quemard, A., Blanchard, J.S., Jacobs, W.R., Jr, and Sacchettini, J.C. (1995) Crystal structure and function of the isoniazid target of Mycobacterium tuberculosis. Science 267: 1638–1641.

Dover, L.G., Alahari, A., Gratraud, P., Gomes, J.M., Bhowruth, V., Reynolds, R.C., et al. (2007) EthA, a common activator of thiocarbamide-containing drugs acting on different mycobacterial targets. Antimicrob Agents Chemother 51: 1055–1063.

Dubos, R.J., and Middlebrook, G. (1947) Media for tubercle bacilli. Am Rev Tuberc 56: 334–345.

Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Laloo, U., et al. (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 368: 1575–1580.

Hazen, M.H., Brimacombe, M., Bobadilla del Valle, M., Cavatore, M., Guerrero, M.I., Varma-Basil, M., et al. (2006) Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant Mycobacterium tuberculosis. Antimicrob Agents Chemother 50: 2640–2649.

Koledin, T., Newton, G.L., and Fahey, R.C. (2002) Identification of the mycothiol synthase gene (mshD) encoding the acetyltransferase producing mycothiol in actinomycetes. Arch Microbiol 178: 331–337.

Larsen, M.H., Vilchez, C., Kremer, L., Besra, G.S., Parsons, L., Salfinger, M., et al. (2002) Overexpression of inhA, but not kasA, confers resistance to isoniazid and ethionamide in Mycobacterium smegmatis. Mol Microbiol 46: 453–466.

Leonardo, M.R., Dailly, Y., and Clark, D.P. (1996) Role of NAD in regulating the adhE gene of Escherichia coli. J Bacteriol 178: 6013–6018.

Lund, O., Nielsen, M., Lundegaard, C., and Worning, P. (2002) CPHmodels 2.0: X3M a Computer Program to Extract 3D Models. In CASP5 Conferencea102.

Marrakchi, H., Laneelle, G., and Quemard, A. (2000) InhA, a target of the antituberculous drug isoniazid, is involved in a mycobacterial fatty acid elongation system, FAS-II. Microbiology 146 (Part 2): 289–296.

Martin, A., Morcillo, N., Lemus, D., Montoro, E., Telles, M.A., Simboli, N., et al. (2005) Multicenter study of MTT and resazurin assays for testing susceptibility to first-line anti-tuberculosis drugs. Int J Tuberc Lung Dis 9: 901–906.

Middlebrook, G. (1954) Isoniazid resistance and catalase activity of tubercle bacilli. Am Rev Tuberc 69: 471–472.

Middlebrook, G., Cohn, M.L., and Schaefer, W.B. (1954) Studies on isoniazid and tubercle bacilli. III. The isolation, drug-susceptibility, and catalase-testing of tubercle bacilli from isoniazid-treated patients. Am Rev Tuberc 70: 852–872.

Miesel, L., Weisbrod, T.R., Marcinkeviciene, J.A., Bittman, R., and Jacobs, W.R., Jr (1998) NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in Mycobacterium smegmatis. J Bacteriol 180: 2459–2467.

Morlock, G.P., Methochuk, B., Sikes, D., Crawford, J.T., and Cooksey, R.C. (2003) ethA, inhA, and katG loci of ethionamide-resistant clinical Mycobacterium tuberculosis isolates. Antimicrob Agents Chemother 47: 3799–3805.

Newton, G.L., and Fahey, R.C. (2002) Mycothiol biochemistry. Arch Microbiol 178: 388–394.

Newton, G.L., Arnold, K., Price, M.S., Sherrill, C., Delcardayre,
S.B., Aharonowitz, Y., et al. (1996) Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. J Bacteriol 178: 1990–1995.

Newton, G.L., Unson, M.D., Anderberg, S.J., Aguilera, J.A., de Oh, N.N.I., Cardayre, S.B., et al. (1999) Characterization of Mycobacterium smegmatis mutants defective in 1-D-myo-inositol-2-amino-2-deoxy-alpha-D-glucopyranoside and mycothiol biosynthesis. Biochem Biophys Res Commun 255: 239–244.

Newton, G.L., Av-Gay, Y., and Fahey, R.C. (2000a) A novel mycothiol-dependent detoxification pathway in mycobacteria involving mycothiol S-conjugate amidase. Biochemistry 39: 10739–10746.

Newton, G.L., Av-Gay, Y., and Fahey, R.C. (2000b) N-acetyl-1-D-myo-inositol-2-amino-2-deoxy-alpha-D-glucopyranoside deacetylase (MshB) is a key enzyme in mycothiol biosynthesis. J Bacteriol 182: 6958–6963.

Newton, G.L., Koledin, T., Gorovitz, B., Rawat, M., Fahey, R.C., and Av-Gay, Y. (2003) The glycosyltransferase gene encoding the enzyme catalyzing the first step of mycothiol biosynthesis (mshA). J Bacteriol 185: 3476–3479.

Newton, G.L., Ta, P., Bzymek, K.P., and Fahey, R.C. (2006) Biochemistry of the initial steps of mycothiol biosynthesis. J Biol Chem 281: 33910–33920.

Piatak, A.S., Telenti, A., Murray, M.R., El-Hajj, H., Jacobs, W.R., Jr, Kramer, F.R., and Alland, D. (2000) Genotypic analysis of Mycobacterium tuberculosis in two distinct populations using molecular beacons: implications for rapid susceptibility testing. Antimicrob Agents Chemother 44: 103–110.

Quemard, A., Laneelle, G., and Lacave, C. (1992) Mycolic acid synthesis: a target for ethionamide in mycobacteria? Antimicrob Agents Chemother 36: 1316–1321.

Quemard, A., Sacchettini, J.C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W.R., Jr, and Blanchard, J.J. (1995) Enzymatic characterization of the target for isoniazid in Mycobacterium tuberculosis. Biochemistry 34: 8235–8241.

Ramawamy, S.V., Reich, R., Dou, S.J., Jaspere, L., Pan, X., Wanger, A., et al. (2003) Single nucleotide polymorphisms in genes associated with isoniazid resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 47: 1241–1250.

Rawat, M., Kovacevic, S., Billman-Jacobe, H., and Av-Gay, Y. (2003) Inactivation of mshB, a key gene in the mycothiol biosynthesis pathway in Mycobacterium smegmatis. Microbiology 149: 1341–1349.

Rawat, M., Johnson, C., Cadiz, V., and Av-Gay, Y. (2007) Comparative analysis of mutants in the mycothiol biosynthesis pathway in Mycobacterium smegmatis. Biochem Biophys Res Commun 363: 71–76.

Rozwarski, D.A., Grant, G.A., Barton, D.H., Jacobs, W.R., Jr, and Sacchettini, J.C. (1998) Modification of the NADH of the isoniazid target (inhA) from Mycobacterium tuberculosis. Science 279: 98–102.

San, K.Y., Bennett, G.N., Berrios-Rivera, S.J., Vadali, R.V., Yang, Y.T., Horton, E., et al. (2002) Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in Escherichia coli. Metab Eng 4: 182–192.

Sareen, D., Newton, G.L., Fahey, R.C., and Buchmeier, N.A. (2003) Mycothiol is essential for growth of Mycobacterium tuberculosis Erdman. J Bacteriol 185: 6736–6740.

Shah, N.S., Wright, A., Bai, G.H., Barrera, L., Boulahbal, F., Martin-Casabona, N., et al. (2007) Worldwide emergence of extensively drug-resistant tuberculosis. Emerg Infect Dis 13: 380–387.

Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., et al. (1991) New use of BCG for recombinant vaccines. Nature 351: 456–460.

Takayama, K., Wang, L., and David, H.L. (1972) Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of Mycobacterium tuberculosis. Antimicrob Agents Chemother 2: 29–35.

Telenti, A., Honore, N., Bernasconi, C., March, J., Ortega, A., Heym, B., et al. (1997) Genotypic assessment of isoniazid and rifampin resistance in Mycobacterium tuberculosis: a blind study at reference laboratory level. J Clin Microbiol 35: 719–723.

Vannelli, T.A., Dykman, A., and Ortiz de Montellano, P.R. (2002) The antituberculosis drug ethionamide is activated by a flavoprotein monooxygenase. J Biol Chem 277: 12824–12829.

Vetting, M.W., Frantom, P.A., and Blanchard, J.S. (2008) Structural and enzymatic analysis of MshA from Corynebacterium glutamicum: substrate-assisted catalysis. J Biol Chem 283: 15834–15844.

Vilcheze, C., and Jacobs, W.R., Jr (2007a) Isolation and analysis of Mycobacterium tuberculosis mycolic acids. Current Protocols in Microbiology: Unit 10A.13.

Vilcheze, C., and Jacobs, W.R., Jr (2007b) The mechanism of isoniazid killing: clarity through the scope of genetcs. Annu Rev Microbiol 61: 35–50.

Vilcheze, C., Weisbrod, T.R., Chen, B., Kremer, L., Hazbon, M.H., Wang, F., et al. (2005) Altered NADH/NAD+ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria. Antimicrob Agents Chemother 49: 708–720.

Vilcheze, C., Wang, F., Arai, M., Hazbon, M.H., Colangeli, R., Kremer, L., et al. (2006) Transfer of a point mutation in Mycobacterium tuberculosis inhA resolves the target of isoniazid. Nat Med 12: 1027–1029.

Wang, F., Langley, R., Gulten, G., Dover, L.G., Besra, G.S., Jacobs, W.R., Jr, and Sacchettini, J.C. (2007) Mechanism of thioamide drug action against tuberculosis and leprosy. J Exp Med 204: 73–78.

Wilming, M., and Johnsson, K. (1999) Spontaneous formation of the bioactive form of the tuberculosis drug isoniazid. Angew Chem Int Ed Engl 38: 2588–2590.

Winder, F.G., and Collins, P.B. (1970) Inhibition by isoniazid of synthesis of mycolic acids in Mycobacterium tuberculosis. J Gen Microbiol 63: 41–48.

Winder, F.G., Collins, P.B., and Whelan, D. (1971) Effects of ethionamide and isoxyl on mycolic acid synthesis in Mycobacterium tuberculosis BCG. J Gen Microbiol 66: 379–380.

Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992) The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature 358: 591–593.

Zignol, M., Hosseini, M.S., Wright, A., Weezenbeek, C.L., Nunn, P., Watt, C.J., et al. (2006) Global incidence of multidrug-resistant tuberculosis. J Infect Dis 194: 479–485.
Supplementary material

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