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Resistance to Isoniazid and Ethionamide in *Mycobacterium tuberculosis*: Genes, Mutations, and Causalities

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Abstract

Isoniazid (INH) is the cornerstone of tuberculosis (TB) chemotherapy, used for both treatment and prophylaxis of TB. The antimycobacterial activity of INH was discovered in 1952, and almost as soon as its activity was published, the first INH-resistant *Mycobacterium tuberculosis* strains were reported. INH and its structural analog and second-line anti-TB drug ethionamide (ETH) are pro-drugs. INH is activated by the catalase-peroxidase KatG, while ETH is activated by the monooxygenase EthA. The resulting active species reacts with NAD^+ to form an INH-NAD or ETH-NAD adduct, which inhibits the enoyl ACP reductase InhA, leading to mycolic acid biosynthesis inhibition and mycobacterial cell death. The major mechanism of INH resistance is mutation in *katG,* encoding the activator of INH. One specific KatG variant, S315T, is found in 94% of INH-resistant clinical isolates. The second mechanism of INH resistance is a mutation in the promoter region of *inhA* (c-15t), which results in *inhA* overexpression and leads to titration of the drug. Mutations in the *inhA* open reading frame and promoter region are also the major mechanism of resistance to ETH, found more often in ETH-resistant clinical isolates than mutations in the activator of ETH. Other mechanisms of resistance to INH and ETH include expression changes of the drugs' activators, redox alteration, drug inactivation, and efflux pump activation. In this article, we describe each known mechanism of resistance to INH and ETH and its importance in *M. tuberculosis* clinical isolates.

THE EMERGENCE OF DRUG RESISTANCE

Tuberculosis (TB) chemotherapy started in the 1930s with the discovery by Domagk and colleagues of the anti-TB activity of sulfonamides. Since these compounds were very toxic and highly insoluble, analogs were synthesized, leading to the discovery of Tibione (thiacetazone, Fig. 1), a highly effective thiosemicarbazone against *Mycobacterium tuberculosis* (1, 2). In parallel, the natural product streptomycin (SM), discovered by Schatz and Waksman, showed activity against *M. tuberculosis* (3) and was used successfully to treat TB patients. Two new anti-TB drugs were discovered soon after: para-aminosalicylic acid (PAS) in 1946 (4) and isonicotinic acid hydrazine (isoniazid, INH) in 1952 (5, 6). Each drug had activity against *M. tuberculosis;* however, drug-resistant mutants emerged rapidly during clinical trials (7–9). To prevent drug resistance, in 1959, SM, PAS, and INH were combined to form the first successful multidrug, biphasic chemotherapy for TB (10). This combination

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treatment was so impressive that Selman Waksman wrote, “the ancient foe of man, known as consumption, the great white plague, tuberculosis, or by whatever other name, is on the way to being reduced to a minor ailment of man. The future appears bright indeed, and the complete eradication of the disease is in sight” (185, p. 217). Nevertheless, the treatment was long and expensive, and patients often dropped out prior to completing chemotherapy. In 1984, a new short-course treatment was established that showed improved efficacy and patient compliance; the drug regimen consisted of two months on INH, rifampicin (RIF), pyrazinamide (PZA), and ethambutol, followed by four months on INH and RIF only.

Despite this successful chemotherapy, the rate of multidrug-resistant (MDR) *M. tuberculosis* strains, defined as strains resistant to INH and RIF, started to increase as early as 1985. Nowadays, the World Health Organization (WHO) estimates that 3.7% of new TB cases and 20% of previously treated TB cases are caused by MDR-TB (11). The highest incidence of MDR-TB (up to 76%) is found in Russia and the former Soviet republics. Extensively drug-resistant (XDR)-TB, defined as TB strains resistant to INH, RIF, fluoroquinolones, and one second-line injectable drug, is found in up to 9% of MDR-TB cases and has been reported in at least 84 countries so far. Furthermore, strains of *M. tuberculosis* that are resistant to up to 10 TB drugs, referred to as totally drug-resistant (TDR-TB), have been isolated in Europe, Africa, India, and Iran (12). One factor in the emergence and rapid spread of drug resistance is the paucity of rapid diagnostics. While new tools are available to quickly assess drug resistance, these methods are based on known drug resistance mechanisms. Understanding all these mechanisms is key to improving diagnosis and eradicating drug-resistant *M. tuberculosis*. In this article, we will discuss the mechanisms that *M. tuberculosis* developed to become resistant to the first-line anti-TB drug INH and its analog, the second-line anti-TB drug ethionamide (ETH).

MODE OF ACTION OF INH AND ETH

INH

The antimycobacterial activity of INH was published and commercialized simultaneously by three independent pharmaceutical companies: Bayer (Neoteben) (13), Hoffman-La Roche (Rimifon) (6), and Squibb Institute for Medical Research (Nydrazid) (5). INH had been first synthesized 40 years earlier and reported in a doctoral thesis; therefore, none of the pharmaceutical companies could patent the discovery. Fox (6) described the discovery of INH as an attempt to combine the anti-TB activity of nicotinamide, which had been found to arrest *M. tuberculosis* growth in guinea pigs (14), and thiosemicarbazones. By replacing the benzene ring of Tibione by the pyridine ring found in nicotinamide (Fig. 1), *meta*- and para-pyridylaldehyde thiosemicarbazones were synthesized. All the intermediates in the synthesis of these pyridine thiosemicarbazones were tested for activity against TB, and among them, one intermediate, INH, had antimycobacterial activity far superior to any compound at the time (6). Notably, another analog synthesized, Marsilid (1-isonicotinoyl-2-isopropylhydrazine) (Fig. 1), also showed good anti-TB activity. The use of Marsilid as a TB drug was soon discontinued because it induced euphoria in TB patients. However, Marsilid did go on to become the first antidepressant. Interestingly, the earlier synthetic compounds

active to some degree against *M. tuberculosis* share a similar chemical skeleton (Fig. 1), although their modes of action are different.

The mechanism of action of INH has been the subject of intensive research and controversies since its discovery in 1952. From 1953 to 1980, multiple modes of action for INH were proposed: INH was thought to interfere with cell division (15), pyridoxal-dependent metabolic pathway(s) (16), lipid biosynthesis (17), fatty acid biosynthesis (18), nucleic acid biosynthesis (19), glycerol conversion to hexose phosphate (20), NAD^+ biosynthesis (21), and NADH dehydrogenase activity (22–23). A major breakthrough in understanding the mechanism of action of INH came with the study by Winder and Collins (186) in which they showed that INH inhibited synthesis of mycolic acids, long-chain α-alkyl β-hydroxy fatty acids that are a crucial component of the mycobacterial cell wall. The effect of INH on mycolic acids has been subsequently confirmed by numerous researchers. Among them, Takayama and coworkers were the first to demonstrate that inhibition of mycolic acid biosynthesis by INH correlated with cell death (24), accumulation of long-chain fatty acids (25), and inhibition of C24 and C26 monounsaturated fatty acid biosyntheses (26). Takayama and colleagues concluded that the mode of action of INH involved a desaturase required for the biosynthesis of these unsaturated fatty acids (26).

INH, like other TB drugs (ETH, PZA, isoxyl, thiacetazone) is a pro-drug. The catalase peroxidase KatG (encoded by *Rv1908c)* activates INH to form a hypothetical isonicotinoyl anion or radical (27–30). This entity reacts with NAD^+ to yield an INH-NAD adduct, which binds to the active site of the NADH-dependent enoyl-ACP reductase InhA (Rv1484) (Fig. 2) (31). This enzyme reduces monounsaturated acyl-ACP to acyl-ACP (32, 33) and is part of the fatty acid synthase type II (FASII) (34). FASII elongates fatty acids up to 56 carbons long where they are derivatized and coupled to a C24-C26 fatty acid generated by FASI to form mycolic acids. The INH-NAD adduct binds to and inhibits InhA (28, 35, 36), leading to disruption of mycolic acid biosynthesis and cell death (37, 38).

ETH

2-Ethylthioisonicotinamide, ETH (Fig. 1), is a structural analog of INH. ETH was first synthesized in 1956 by a French team trying to improve on the antimycobacterial properties of thioisonicotinamide (39). Grumbach and coworkers found that ETH was more active than SM but less so than INH against *M. tuberculosis*. However, ETH was also active against INH-, PAS-, and SM-resistant *M. tuberculosis* strains. ETH was shown to be efficacious in combination with PZA and with or without INH in a clinical trial to treat TB patients infected with INH- and SM-resistant strains (40, 41). Nowadays, ETH is a second-line drug, mostly used to treat MDR-TB cases in South Africa.

Similar to INH (42, 43), *M. tuberculosis* treated with ETH loses its acid fastness (39) and its ability to synthesize mycolic acids (44). ETH is also a pro-drug, activated by the NADPH-specific flavin adenine dinucleotide-containing monooxygenase EthA (also called EtaA, encoded by *Rv3854c)* (45–48). Once activated, the mode of action of ETH is very similar to INH (Fig. 2). The active form of ETH reacts with NAD^+ to yield an ETH-NAD adduct (49), which inhibits InhA, leading to mycolic acid biosynthesis inhibition. Interestingly, while

KatG only activates INH, EthA activates two other second-line anti-TB drugs: Tibione (thiacetazone) and isoxyl (46, 50).

MECHANISMS OF RESISTANCE TO INH AND ETH

Drug resistance in mycobacteria is due to the acquisition of mutations or efflux pump activation, not due to the acquisition of resistance plasmids or transposons, common resistance mechanisms in other bacterial species. The main mechanisms of resistance to INH and ETH can be divided into two categories. First, prevention of the activation of INH and ETH can be obtained by mutating the activators of the drugs *katG* and *ethA,* respectively, or by mutating regulators of their expression. For example, the katG(S315T) mutation is found in up to 94% of INH-resistant *M. tuberculosis* clinical isolates. Second, the inhibition of InhA by the INH-NAD or ETH-NAD adduct can be overcome by mutations in *inhA* or its promoter region. Other mechanisms of resistance exist such as drug inactivators, redox alteration, and efflux pumps. We will first describe the mechanisms of coresistance to INH and ETH and then the mechanisms of resistance specific to each drug.

Common Mechanisms of Resistance to INH and ETH

Clinical isolates coresistant to INH and ETH were isolated from TB patients who had received INH but had never been treated with ETH (51–53). This conundrum led to the hypothesis that INH and ETH shared a common mechanism of resistance, a hypothesis that could not be tested until a plasmid transformation system was developed for mycobacteria (54, 55). It would take another three decades to discover that INH and ETH target the same enzyme in *M. tuberculosis:* the enoyl-ACP reductase InhA (56). Other mechanisms of coresistance to INH and ETH have been discovered. These mechanisms along with resistance mechanisms due to *inhA* mutations are listed below.

Alteration of InhA, the target of INH and ETH—The target of INH and ETH was discovered by isolating a *Mycobacterium smegmatis* mutant coresistant to INH and ETH (56). A genomic DNA cosmid library of this *M. smegmatis* mutant and of drug-susceptible *Myco-bacterium avium, Mycobacterium bovis, M. smegmatis,* and *M. tuberculosis* strains was constructed and transformed into *M. smegmatis.* A single open reading frame (ORF) was found to be sufficient to confer coresistance to INH and ETH in *M. smegmatis* and was named *inhA.* These experiments demonstrated that a single amino acid mutation in *inhA*(S94A) or overexpression of *inhA* conferred coresistance to INH and ETH in mycobacteria. To prove that *inhA* inactivation was sufficient to lead to death in a manner similar to INH action, a temperature-sensitive mutant in *inhA* was isolated (37). Heat-inactivation of InhA(V238F) mimicked the effects of INH in *M. tuberculosis* described by Takayama and colleagues: inhibition of mycolic acid biosynthesis (24), alteration of the bacterium morphology (57), accumulation of long-chain fatty acids (25), and cell death (24), demonstrating that inhibition of InhA alone reproduced the biochemical characteristics of INH treatment of *M. tuberculosis*. Moreover, the S94A mutation identified in the INH- and ETH-resistant *M. smegmatis* mutant was transferred into wild-type *M. tuberculosis* by specialized linkage transduction, and the resulting strain was at least five times more resistant to INH and ETH than wild-type *M. tuberculosis* (38). This combined set of data

confirms that InhA is the main target and the main mechanism of coresistance to INH and ETH.

The mechanism by which the S94A mutation leads to resistance to INH and ETH has been well studied. InhA is an NADH-dependent enoyl-ACP reductase, and the binding of the enoyl substrate to InhA is not disturbed by the S94A mutation; however, the mutation results in a 5-fold increase in the K_M for the InhA cofactor NADH (32). On the other hand, the ability of the INH-NAD adduct to inhibit InhA(S94A) is markedly reduced, because the IC_{50} and K_i are 17 and 30 times higher, respectively, for the mutated protein. Comparison of the crystal structures of InhA(S94A) to wild-type InhA revealed the loss of a water molecule and disruption of a hydrogen bonding network in the mutated protein, which was enough to reduce the binding of the INH-NAD adduct (38). Others disputed this conclusion and hypothesized that InhA interacts with FASII enzymes and that this interaction is perturbed by the S94A mutation, resulting in INH resistance (58). Overexpression of *inhA* is also a common factor of INH and ETH resistance in clinical isolates. The c-15t base pair change in the *inhA* regulatory region increases *inhA* mRNA levels by 20-fold, resulting in the overexpression of InhA. This leads to a titration of INH or ETH and consequently an eight-fold increase in INH and ETH MICs in *M. tuberculosis* (38).

Numerous point mutations in *inhA* and its promoter region have been identified in INH- and ETH-resistant *M. tuberculosis* clinical isolates (Tables 1 and 2); however, no base pair insertions or deletions have been observed. Mutations in the *inhA* promoter and ORF regions are associated with low-level resistance to INH, even when strains contained mutations in both regions (MIC <1 mg/liter) (59). The c-15t mutation in the promoter region of *inhA* is found in up to 35% of INH-resistant and 55% of ETH-resistant clinical isolates, but never in INH- or ETH-sensitive strains. This mutation was also overly represented in XDR-TB cases in South Africa. In a study on clinical isolates from the Western Cape Province, South Africa, the c-15t mutation was present in 30% of strains mono-resistant to INH, 48% of MDR-TB, and 85% of XDR-TB (60), suggesting that this mutation could be a marker for XDR-TB. Interestingly, in a survey of the Eastern Cape Province clinical isolates, the c-17t *inhA* promoter mutation was the predominant genetic modification present in 83% of XDR-TB cases. The combined *inhA* promoter mutations (at positions −8, −15, and −17) were found in 92% of the XDR-TB cases versus 62% of the MDR-TB cases in the Eastern Cape Province (60).

inhA is an essential gene; therefore, mutations in the coding region of *inhA* are rare. About 15 mutations in *inhA* have been identified in INH-resistant clinical isolates, although two of them were also found in INH-sensitive strains (Table 1). I21T, S94A, and I95P are the only amino acid changes found in both INH- and ETH-resistant clinical isolates. The first mutation identified in *inhA* in *M. smegmatis,* resulting in the S94A variant, has since been found in INH-resistant *M. tuberculosis* clinical isolates with no other mutation present in *katG* or the *fabG-inhA* intergenic region (59, 61). This confirms that the S94A mutation is sufficient to confer INH and ETH resistance in *M. tuberculosis* clinical isolates.

While mutations in the *inhA* promoter region can represent up to 35% of the INH-resistant cases, and mutations in the *inhA* gene are rare in INH-resistant clinical isolates, this is the

predominant region where mutations are found in ETH-resistant clinical isolates. In one study (62), 62% of the ETH-resistant clinical isolates had mutations in *inhA* (gene and/or promoter region), while 47% had mutations in *ethA.* The *inhA* promoter mutation c-15t was therefore proposed as a marker for ETH resistance (63).

Alteration of redox potential—*M. smegmatis* and *M. bovis* BCG mutants coresistant to INH and ETH were isolated in *in vitro* experiments from nonmutagenized independent cultures. The mutants had mutations in *ndh (Rv1854c),* a gene encoding a type II NADH dehydrogenase, which oxidizes NADH into NAD^+. In *M. smegmatis,* the *ndh* mutants contained single base pair changes resulting in amino acid changes and a pleitropic phenotype: INH resistance, ETH resistance, temperature sensitivity, and for some mutants, Ser/Gly auxotrophy (64, 65). In *M. bovis* BCG, the mutants had either single base pair changes or insertions (65). The *ndh* mutants lost up to 95% of their Ndh activity compared to wild type and had higher levels of NADH, while their NAD^+ levels were similar to wild type. An increase in NADH concentration was shown to prevent the binding of the INH-NAD adduct to InhA by acting as a competitive inhibitor, leading to INH resistance (65).

In *M. tuberculosis* clinical isolates, *ndh* mutations have been found in both INH-sensitive and INH-resistant strains at a very low rate (66) (Table 1). However, two studies from Singapore and Brazil identified *ndh* mutations in 8 to 10% of INH-resistant *M. tuberculosis* clinical isolates and found no *ndh* mutations in their INH-sensitive clinical isolate strains (67, 68). The R13C mutation was found in a strain containing the *katG* (S315T) mutation, so the contribution to the INH resistance of this *ndh* mutation is uncertain (67). The *ndh* (T110A) mutation was only associated with an *ahpC* mutation resulting in the (T5I) variant (68). However, in that study, *katG* was only partially sequenced, and *inhA* was not (68, 69), so the INH resistance in that isolate may or may not be due to the *ndh*(T110A) mutation. The R268H mutation is the only mutation so far to have been identified in two independent studies and only in INH-resistant strains, but it was associated with *katG, inhA,* or *ahpC* mutations (66).

M. tuberculosis has an additional NADH dehydrogenase named *ndhA (Rv0392c).* Therefore, mutations in one NADH dehydrogenase such as *ndh* might not alter the redox balance in *M. tuberculosis* and might not lead to INH and/or ETH resistance as long as the second NADH dehydrogenase is functional. *ndh* and *ndhA* are also present in *M. bovis* BCG, but *M. bovis ndhA* codes for a single amino acid change (V241A) relative to *M. tuberculosis,* which might explain why INH- and ETH-resistant *ndh* mutants were isolated in that strain. For that reason, *ndh* mutations might not be a mechanism of resistance to INH and ETH in *M. tuberculosis* unless both NADH dehydrogenase genes are mutated.

Alteration in mycothiol biosynthesis—Mutations in *ndh* in INH-resistant *M. bovis* BCG mutants were isolated *in vitro* by plating nonmuta-genized, independent *M. bovis* BCG cultures on plates containing both INH and ETH to avoid mutants carrying mutations in the activator of INH or ETH. When the same experiment was repeated in *M. tuberculosis* H37Rv or the virulent *M. bovis* Ravenel strain, *ndh* mutants were not obtained. Instead, all of the INH- and ETH-resistant *M. tuberculosis* mutants had mutations in *mshA* (*Rv0486*) (70), a gene encoding a glycosyl transferase involved in the biosynthesis of mycothiol (N-

acetylcysteine glucosamine inositol, MSH), while the *M. bovis* mutants coresistant to INH and ETH carried mutations either in *mshA* or in *mshC (Rv2130c)* encoding the cysteine ligase of the mycothiol biosynthesis (71). Five enzymes are required to synthesize mycothiol: MshA, MshA2, MshB (Rv1170), MshC, and MshD (Rv0819) (72). Mycothiol is the major thiol and the main reducing and detoxifying agent in mycobacteria (72), yet the role of mycothiol during infection is ambiguous since mycothiol-deficient *M. tuberculosis* strains do not have a growth defect *in vivo* (70). Eight *in vitro M. tuberculosis mshA* mutants were isolated containing a single base pair modification in *mshA,* resulting in amino acid changes, stop codons, and frameshifts, all of which caused a drastic decrease in mycothiol levels (from 83 to 99.9%). The mutants had different levels of resistance to INH (2-fold to >10-fold) and ETH (4- to 8-fold increase). Complementation with wild-type *M. tuberculosis mshA* restored ETH susceptibility but not INH susceptibility in all the mutants. Interestingly, deletion of *mshA* in *M. tuberculosis* led to a strain that did not produce mycothiol and was highly resistant to ETH but fully sensitive to INH (70). This suggests that mycothiol is mostly involved in ETH resistance and might play a role in ETH activation. The observed INH resistance in *M. tuberculosis mshA* point mutants and INH susceptibility in *M. tuberculosis AmshA* strains might also indicate that *mshA* is required for INH resistance in mycothiol-deficient strains.

In *M. smegmatis,* a 4- to 8-fold increase in INH and ETH resistance was obtained when *mshA* or *mshC* was deleted, while deletion of *mshB* resulted in a strain resistant only to ETH, and deletion of *mshD* had no effect on INH or ETH resistance (73). *M. smegmatis* mycothiol mutants obtained from chemical mutagenesis or transposon insertion had slightly different INH and ETH resistance patterns (74). The role of mycothiol deficiency in INH resistance might be species-dependent. So far, we can only conclude that mutations in *mshA* will result in high-level ETH resistance and at most low-level INH resistance in *M. tuberculosis.*

In a highly INH- and ETH-resistant clinical isolate, a double mutation in *mshA* (V171G, A187V) was found (62). That strain had the *katG*(S315T) mutation to account for the INH resistance but no other mutation to explain its resistance to ETH. However, the *mshA* (A187V) mutation is present in wild-type *M. tuberculosis* Beijing strain. Other *mshA* mutations have also been found in drug-sensitive mycobacterial strains. The N111S mutation is present in *M. tuberculosis* Erdman and Haarlem strains (70, 75), while the *M. bovis* Ravenel and *M. bovis* ATCC19210 strains carry a g316a base pair change in *mshA,* resulting in a G106R amino acid change (71, 75). Mycothiol genes such as *mshA* and *mshC* should be added to the list of candidates responsible for ETH resistance in clinical isolates, although correlation between *mshA* mutations and ETH resistance should be carefully analyzed since mutations in *mshA* might not lead to drug resistance in *M. tuberculosis.*

Degradation of the INH-NAD or ETH-NAD adduct—In a recent study, Wang and colleagues suggested that the NADH pyrophosphatase NudC (Rv3199c), an enzyme from the NAD^+ recycling pathway, could hydrolyze the INH-NAD or ETH-NAD adduct, leading to INH and ETH resistance (76). *M. smegmatis* and *M. bovis* BCG NudC are functional enzymes, while *M. tuberculosis* H37Rv NudC has a point mutation (Q237P) that renders the enzyme inactive. The authors demonstrated that NudC from *M. smegmatis* and *M. bovis*

BCG could release the adenosine mono-phosphate group from the INH-NAD and ETH-NAD adducts and that overexpression of *M. smegmatis* or *M. bovis* BCG *nudC* resulted in at least a 10-fold increased resistance to INH and ETH, while deletion of *nudC* rendered the strains more sensitive to the drugs. A small portion of *M. tuberculosis* clinical isolates (2%) were found to have the glutamine residue at position 237, suggesting that in these *M. tuberculosis* strains, NudC might be capable of hydrolyzing the adducts. However, no transfer of mutation was performed to prove that this mutation is sufficient to confer INH and ETH resistance in *M. tuberculosis.* The role of *nudC* in INH and/or ETH resistance in *M. tuberculosis* clinical isolates remains to be determined.

Mechanisms of Resistance Specific to INH

Alterations in KatG, the activator of INH—The first mutants isolated in *in vitro* cultures that were highly resistant to INH had the characteristic of being catalase-negative and avirulent in guinea pigs (77, 78). Winder hypothesized that the loss of catalase activity might imply that INH was activated by a catalase to yield some highly reactive species (79). The relationship between INH resistance and the catalase-negative phenotype was elucidated many years later when a highly INH-resistant strain, BHI, a mutant of *M. smegmatis* mc^2155 (55), was complemented with an *M. tuberculosis* library (30). INH susceptibility was restored in BHI by the introduction of a single gene, *katG (Rv1908c),* encoding a catalase-peroxidase. Furthermore, the authors also found *katG* deletion or mutations in INH-resistant *M. tuberculosis* clinical isolates and demonstrated that transformation of these INH-resistant isolates with a wild-type copy of *katG* restored INH susceptibility (30, 80).

In INH-resistant *M. tuberculosis* clinical isolates, more than 300 mutations in *katG* have been identified throughout the ORF (Table 3). Complete deletion of the gene has been found in clinical isolates, including the first INH-resistant *M. tuberculosis* mutant identified in the Zhang study (30); it has subsequently been identified in other studies (Table 3). Point mutations causing a single amino acid substitution or premature termination, frameshift mutations after addition or deletion of bases, and partial or complete deletion of the gene have been identified. The incidence of *katG* mutations differs between geographical regions but represents at least 30% and up to 95% of INH-resistant clinical isolates.

The most frequent *katG* mutation is at codon S315, where each base (AGC) can be mutated to produce a Thr, Asn, Arg, Ile, Gly, or Leu residue. The S315T mutation can be found in up to 94% of the INH-resistant clinical isolates (81). Two independent biochemical analyses reported that KatG(S315T) has catalase-peroxidase activities, yet its ability to oxidize INH was significantly reduced (82, 83). Biochemical analyses of other KatG mutants showed a wide range of catalase-peroxidase and INH oxidase activities (Table 4). Nevertheless, there is a link between INH oxidase activity and INH resistance. *M. tuberculosis* strains with KatG proteins deficient in INH oxidase activity were highly resistant to INH, while *katG* mutants with INH oxidase activity close to wild-type levels had only 2- to 4-fold increases in the MIC for INH. However, the level of INH resistance might not be defined by location of a mutation: S315T, W341G, G494D, and R595STOP variants are highly resistant to INH (84), while L141F, E553K, and F658V variants are associated with low-level INH resistance (84).

A very common polymorphism, R463L, is often found associated with other *katG* mutations and is more likely to be present in INH-sensitive strains than in INH-resistant strains (85).

The high-level resistance to INH associated with *katG* (S315T) (MIC >1 mg/liter) was reported to be specific to the Ser→Thr amino acid change (59, 86). Brossier and colleagues found katG(S315N) only in low-level INH-resistant clinical isolates (62). Curiously, KatG(S315N) had been shown to prevent the formation of the INH-NAD adduct, suggesting that INH cannot be activated by KatG(S315N), which conflicts with the above mentioned results (87). In a different study, a clinical isolate carrying *katG*(S315N) and *inhA*(c-15t), which is associated with low-level resistance to INH (see above), had an MIC for INH of >256 mg/liter (88). This was the same level of resistance found in clinical isolates where *katG* was missing or the mutation resulted in early termination of the protein (Q434STOP) (88). Furthermore, the INH-resistant clinical isolate containing only the *inhA* promoter mutation had an MIC of 0.19 mg/liter, confirming the low-level INH resistance associated with this mutation. This suggests that *katG*(S315N) might be associated with a high level of INH resistance in clinical isolates.

One of the first studies on the isolation of INH-resistant *M. tuberculosis* mutants *in vitro* found that the mutants could be classified into catalase-negative and highly INH-resistant or catalase-positive and weakly INH-resistant (MIC <10 mg/liter) (89). Catalase-negative mutants were unable to grow in guinea pigs and rabbits, while catalase-positive mutants grew relatively well *in vivo* (90). The relationship between catalase activity and fitness of an INH-resistant strain *in vivo* has been investigated. Pym and colleagues demonstrated that the INH-resistant *M. tuberculosis* KatG(T275P) variant had no detectable catalase peroxidase activity and was highly attenuated in a mouse model of infection, while *M. tuberculosis* carrying the KatG(S315T) variant was found to be fully virulent in mice, to have no bacterial fitness cost, and to be fully transmissible (91). Consequently, the S315T variant is more often found in MDR-TB patients than in INH mono-resistant clinical isolates (86) and might be related to the higher transmission capabilities of this particular strain (92). A recent study of TB patients showed that those infected with non-*katG* INH-resistant strains (such as *inhA*[c-15t]) were more likely to exhibit sputum conversion after 1 month than those infected with *katG* mutant strains (93). Transmissibility, virulence, and response to chemotherapy seem to be affected by *katG* mutations.

Alterations of *katG* expression—*katG* is cotranscribed with its negative regulator *furA (Rv1909c),* a gene encoding a ferric uptake regulation protein (94, 95). Deletion of *furA* in *M. tuberculosis* results in overexpression of *katG* and hypersusceptibility to INH (94). Mutations have been identified in *furA* as well as in the 38-bp region between *furA* and *katG* (Table 1). To assess the role of these mutations, Ando and colleagues constructed isogenic strains containing the mutations found in the intergenic region (g-12a, a- 10c, g-7a) or in the *furA* coding sequence (A14V). *katG* expression in the g-12a strain (intergenic region) was only slightly lower than wild-type levels, and the A14V variant had no effect on KatG levels (96); however, the strains with the a-10c or g-7a mutation exhibited an 80% reduction in *katG* expression. This reduction was associated with a decrease in INH oxidase activity and a 2- to 4-fold increase in INH resistance, suggesting that the *furA-katG* intergenic region should be examined in low-level INH-resistant *M. tuberculosis* clinical strains.

Transcriptional analyses indicate that *katG* is also regulated by the sigma factor *sigI* (*Rv1189*)(97). *M. tuberculosis* Δ*sigI* had decreased catalase capabilities and was more resistant to INH than wild-type *M. tuberculosis in vitro* and *in vivo.* Furthermore, overexpression of *sigI* increased the susceptibility of *M. tuberculosis* to INH by 2-fold. Mutations in *sigI* could therefore be another factor that modulates *katG* expression and induces low-level INH resistance in *M. tuberculosis* clinical isolates.

Compensatory mutations—The activity of INH against mycobacterial species is very specific. Other bacteria such as *Escherichia coli* and *Salmonella typhimurium* are not inhibited by a high dose of INH (500 mg/liter or higher; the MIC for *M. tuberculosis* is 0.05 mg/liter). Yet when *E. coli* and *S. typhimurium* have a deficient oxidative stress response regulator, encoded by *oxyR,* the strains become more sensitive to INH (MIC <50 mg/liter) (98). *M. tuberculosis oxyR'* is nonfunctional, because the coding region contains multiple frameshifts and deletions. Downstream of *oxyR'* is *ahpC (Rv2428),* which encodes an alkyl hydroperoxide reductase. Up to 29% of INH-resistant clinical isolates contain mutations in the *oxyR'-ahpC* region (99). Mutations in the *oxyR'-ahpC* intergenic region such as g-9a and c-15t have been shown to increase the expression of *ahpC* by 9- and 18-fold, respectively (100). This increase in *ahpC* expression is thought to compensate for the loss of KatG activity occurring in INH-resistant strains, which would render the strains more susceptible to hydrogen and organic peroxides (101) and to prevent further oxidative damage. However, most variant KatG enzymes, and in particular, KatG (S315T), are competent catalase-peroxidases, meaning that the organism is not deficient in its ability to detoxify peroxides or other compounds. With the present knowledge, the role of *ahpC* in INH resistance is a matter of debate. Baker et al. reported that mutations in the *oxyR'-ahpC* region did not contribute to INH resistance since these mutations could be found in 20% of their INH-resistant clinical isolates but also in 8% of their INH-sensitive isolates (102).

Detoxification of INH—In humans, INH is acetylated by the arylamine *N*-acetyltransferase NAT2. The rate of this detoxification reaction varies between individuals, leading to the classification of TB patients as rapid or slow inactivators depending on their NAT activity. An enzyme similar to NAT is expressed in *M. tuberculosis* (103). When *M. tuberculosis nat* was overexpressed in *M. smegmatis,* the resulting strain was more resistant to INH, with an MIC 3-fold higher than wild-type *M. smegmatis,* suggesting that the mycobacterial arylamine *N*- acetyltransferase can acetylate and inactivate INH (104). Mutations in *nat (nhoA/Rv3566c)* have been identified in INH-resistant clinical isolates (Table 1); G67R and G207R variants were found in INH-resistant and INH-sensitive clinical isolates. Biochemical analyses revealed that the K_M for INH N-acetylation of Nat (G207R) was 10 times that of wild-type Nat (103), indicating that the variant Nat protein is mostly unable to acetylate and therefore inactivate INH. Thus, there is no obvious association between *nat* mutations and INH resistance. Nevertheless, deletion of *nat* in *M. bovis* BCG affected the biosynthesis of mycolic acids, glycolipids, and complex lipids as well as survival in mouse macrophages, indicating that *nat* might modulate other factors involved in INH resistance (105).

Genes induced upon INH treatment—When *M. tuberculosis* comes in contact with INH, numerous genes are upregulated, as first evidenced using a method that employed differential expression using customized amplification libraries (DECAL) (106). The availability of the *M. tuberculosis* genome sequence (107) led to the development of microarrays that have been used to explore the response of *M. tuberculosis* to INH and other drug treatments (108). Transcriptional analysis of INH-treated *M. tuberculosis* revealed that *M. tuberculosis* upregulated a set of genes encoding proteins involved in fatty acid biosynthesis *(fabD, acpM, kasA, kasB, accD6; Rv2243–2247),* trehalose dimycolyl transfer *(fbpC, Rv0129c),* fatty acid degradation *(fadE23, fadE24; Rv3139–3140),* peroxidase activity *(ahpC),* transport *(iniB, iniA; Rv0341–0342),* efflux pump *(efpA, Rv2846c),* and unknown functions *(Rv1592c, Rv1772)* (106,108). Since genes that respond to drug treatment could be implicated in the mechanisms of resistance to the drug, Ramaswany and colleagues sequenced all the genes induced by INH except *kasB, fadE23,* and *acpM* in 38 INH-resistant and 86 INH-sensitive clinical isolates (88). The *kasA* operon is composed of *fabD, acpM, kasA, kasB,* and *accD6. Rv2242,* an *srmR* homolog, is located just upstream of the *kasA* operon and was added to this study. Mutations in *kasA, fadE24, Rv1592c,* and *Rv2242* were found in INH-resistant strains carrying *katG* mutations or in INH-sensitive strains. No mutations in *fbpC, fabD, accD6,* or *efpA* were found in INH-resistant clinical isolates. One T4A mutation was found in *Rv1772* in a low-level INH-resistant clinical isolate, with no mutation in the other 19 genes sequenced. This mutation was not found in the INH-sensitive isolates. Another study found a mutation in *efpA* resulting in a variant (E520V) found in INH-resistant strains only but associated with *katG* and *oxyR ‘-ahpC* mutations. In conclusion, most of the mutations identified in these genes (except for *Rv1772)* in INH-resistant clinical isolates were found either in INH-sensitive strains or in combination with other mutations known to confer INH resistance. Their roles in INH resistance cannot be assessed at this point.

KasA is a beta-ketoacyl-ACP synthase that condenses an elongating fatty acyl-ACP with malonyl-ACP and is the first enzyme in the FASII system. KasA was once considered a more likely target of INH than InhA based on the fact that INH treatment of *M. tuberculosis* resulted in the inhibition of mycolic acid biosynthesis and accumulation of the long-chain fatty acid hexacosanoic acid. The accumulation of fatty acids correlated better with the inhibition of a beta-ketoacyl-ACP synthase (KasA) than with the inhibition of an enoyl-ACP reductase (InhA), which should result in the accumulation of enoyl products (unsaturated fatty acids) (109). This biochemical red herring, however, did not consider the work of Takayama and coworkers, who demonstrated that a short exposure (5 min) of *M. tuberculosis* to INH resulted in the accumulation of unsaturated fatty acids, while longer INH exposures led to accumulation of hexacosanoic acid due to the total shutdown of the FASII system (26). Mdluli et al. demonstrated that in INH-treated *M. tuberculosis*, KasA formed a complex with INH and the acyl carrier protein AcpM and identified four variants in KasA (D66N, G269S, G312S, F413L) in INH-resistant clinical isolates (109). The authors thus concluded that KasA was the main target of INH. This conclusion was disproved by the demonstration that the complex between KasA, INH, and AcpM was formed only when InhA was inhibited (110) and by the discovery of the KasA variants D66N, G269S, and G312S in INH-sensitive strains (66). Other *kasA* mutations have been

identified in INH-resistant *M. tuberculosis* clinical isolates; however, these mutations are usually associated with *katG* mutations.

The INH-inducible gene *iniA* was shown to confer tolerance to INH when overexpressed in *M. bovis* BCG but not in *M. tuberculosis*, while deletion of *iniA* rendered *M. tuberculosis* more susceptible to INH (111). *iniA* is in an operon with the INH-inducible genes *iniB* and *iniC.* Immediately upstream of this operon is *Rv0340,* which is transcribed in the same direction. This operon is induced specifically by drugs inhibiting mycobacterial cell wall biosynthesis such as INH and ethambutol (112). The *iniBAC* operon encodes a membrane transporter, but it was shown not to transport INH (111). All the mutations in this cluster (Table 1) were only present in INH-resistant clinical isolates; however, they were always associated with mutations in *katG* and/or *inhA.* Hence, mutations in the *iniBAC* operon may only have a minor role in INH resistance.

Interestingly, the target of INH and ETH, *inhA,* is not upregulated upon INH or ETH treatment of *M. tuberculosis,* which may be an important determinant of successful drug targets.

Overexpression of NAD^+/$NADP^+$-binding enzymes—The INH-sensitive *E. coli oxyR* mutant described above was also used to identify other molecular determinants of INH resistance in *M. tuberculosis*. The *E. coli* mutant was transformed with an *M. tuberculosis* genomic plasmid library and screened for clones that became INH-resistant. Three genes, *glf* (*ceoA*), *ceoB,* and *ceoC*, restored INH resistance in *E. coli*. Each gene contains an NAD^+ binding motif. Only overexpression of *M. tuberculosis glf (Rv3809c),* an NAD^+- and flavin adenine dinucleotide-dependent UDP galactopyranose mutase, led to low-level INH resistance in BCG. However, a binding experiment with radioactive INH showed that Glf did not bind to INH. The authors concluded that upregulation of NAD^+-binding proteins might play a role in INH resistance by either reducing the levels of NAD^+ available for the formation of the INH-NAD adduct or by binding an unknown derivative of INH (113).

The finding that the NADPH-dependent β-ketoacyl-ACP reductase FabG (Rv1483), part of the FASII system, was inhibited *in vitro* by an INH-NADP adduct similarly to InhA (114) led others to investigate whether additional enzymes could be inhibited by this INH- NADP adduct. The enzymatic activity of the *M. tuberculosis* dihydrofolate reductase DHFR (Rv2763c), the target of the broad-spectrum antibiotic trimethoprim, was shown to be inhibited by the *4R*-INH-NADP adduct with subnanomolar affinity (115). Overexpression of *M. tuberculosis dfrA* in *M. smegmatis* increased INH resistance by 2-fold at 30°C (115). However, overexpression of *M. tuberculosis dfrA* in *M. tuberculosis* did not increase resistance to INH (116), and the sequences of 127 INH-resistant clinical isolates revealed no mutation in *dfrA,* suggesting that DHFR is not a marker for INH resistance in *M. tuberculosis* (117). Affinity chromatography identified 16 other proteins that could bind to the INH-NAD or INH-NADP adduct (118), but overexpression of the *M. tuberculosis* genes encoding these proteins did not confer resistance to INH or ETH in *M. smegmatis* (116).

Overexpression of efflux pumps—Active export of drugs from cells by efflux pumps was first described in *E. coli* for tetracycline (119). In mycobacteria, low-level resistance to

tetracycline and other aminoglycosides has been attributed to efflux pumps (120, 121). *iniA,* a gene induced by INH treatment (106), was shown to be a component of an MDR-like efflux pump. Overexpression of *iniA* conferred tolerance to INH, while deletion of *iniA* increased the susceptibility of mycobacteria to INH (111). However, IniA does not pump INH out of the cells.

A microarray study of *M. tuberculosis* clinical isolates resistant to INH, RIF, SM, ethambutol, and ofloxacin revealed that upon INH treatment, expression of several predicted efflux pump genes was upregulated: *Rv1819c, Rv2459, Rv2846, Rv3065, and Rv3728* (122). Furthermore, administration of an efflux pump inhibitor decreased the MIC for INH in these strains by at least 4-fold, suggesting that these efflux pumps may play a role in drug resistance in *M. tuberculosis.*

Rv1217c-1218c, an operon encoding an ATP binding cassette transporter, was shown by RT-qPCR to be overexpressed in MDR- and XDR-TB strains; the overexpression of *Rv1218C* was associated with increased INH resistance (123). However, deletion of *Rv1218c* did not affect the MIC of INH in *M. tuberculosis* (124), nor did deletion of other efflux pumps such as *Rv1877, mmr,* and *mmpL7* (125,126), although overexpression of *mmpL7* conferred resistance to INH (127). Conversely, deletion of *lfrA* led to a 2-fold decrease in the INH MIC (126). Unexpectedly, deletion of *efpA,* which encodes an efflux pump that is specifically induced upon INH treatment of *M. tuberculosis*, resulted in increased resistance to INH and RIF in *M. smegmatis* (126).

In light of these studies, the role of efflux pumps in INH resistance in *M. tuberculosis* needs to be further evaluated in order to assess their importance in the resistance mechanisms.

Mechanisms of Resistance to ETH Only

Alterations of EthA, the activator of ETH—The mechanisms of resistance to ETH are mutations in genes encoding its activator *(ethA),* its target *(inhA),* or the *ethA* regulator *(ethR).* So far, 85 *ethA* mutations have been identified, although some were also found in drug-susceptible or partially ETH-resistant *M. tuberculosis* strains (Table 2). Mutations have been identified throughout the length of the coding region. Approximately two-thirds of the nucleotide changes are missense mutations that result in amino acid changes, while the remaining mutations are insertions, deletions, or nonsense mutations. Unlike the *katG*(S315T) variant, which can be present in up to 94% of the INH-resistant clinical isolates, no dominant *ethA* mutation occurs in ETH-resistant clinical isolates. Morlock and colleagues (61) hypothesized that the lack of cluster or dominant *ethA* mutations in ETH-resistant clinical isolates could be attributed to the presence of numerous monooxygenase homologs in *M. tuberculosis* that could protect the cells against a loss of EthA activity.

In a study by Brossier and colleagues (62), 47 ETH-resistant clinical isolates were analyzed for mutations in *ethA, ethR, inhA, ndh,* or *mshA.* Of these, 22 clinical isolates (47%) had mutations in *ethA,* while 29 strains (62%) had a mutation in *inhA* (promoter region/gene). On average, the proportion of *inhA* mutations in ETH-resistant clinical isolates is 68%, suggesting that this is the main mechanism of ETH resistance in *M. tuberculosis.*

Mutations in *ethR*, the regulator of ethA—*ethA* is negatively regulated by EthR (Rv3855), a transcriptional repressor belonging to the TetR family. The two genes are oriented opposite to each other, separated by a 73-bp intergenic region that contains the *ethA* promoter and to which EthR binds. A strain with a transposon insertion in *ethR* was highly sensitive to ETH, while *ethR* overexpression increased the resistance to ETH (45). Two mutations in *ethR* have been identified (Table 2) in highly ETH-resistant clinical strains, which represent 4% of ETH-resistant clinical strains screened; however, these two strains also contained the c-15t mutation in the promoter region of *inhA,* and one of the two had a mutation in *ethA* as well (62). Since the majority of ETH-resistant clinical isolates have mutations in *ethA* and/or *inhA, ethR* might only play a minor role in ETH resistance in *M. tuberculosis* clinical isolates.

CONCLUSIONS AND THE FUTURE

The mechanisms of resistance to INH and ETH in *M. tuberculosis* are both simple and complex (Fig. 3). The main mechanisms of resistance are mutations in *katG* and *ethA,* the activators of INH and ETH, respectively, preventing the formation of the INH-NAD or ETH-NAD adduct, and mutations in *inhA,* the target of INH and ETH, leading to titration of the drug or reduced binding of the INH-NAD or ETH-NAD adduct to InhA. Numerous mutations in multiple genes have been identified in INH- or ETH-resistant *M. tuberculosis* clinical isolates (Tables 1 to 3). Ultimately, the validation of a mutation responsible for INH or ETH resistance requires transferring the point mutation into wild-type *M. tuberculosis* and measuring the level of resistance. This is far beyond the scope of most studies that describe mutations associated with drug resistance in *M. tuberculosis.*

It is often reported that a certain percentage (up to 30%) of INH-resistant *M. tuberculosis* clinical isolates have no mutation in any of the genes studied, leading some to conclude that there is still much more to discover about INH and ETH mode of action and resistance. Yet most studies sequenced only a small fraction of the genes known to confer INH resistance such as *katG* and *inhA,* looking only at the region around codon 315 of *katG* or the regulatory region of *inhA.* However, more than 300 mutations have been identified in *katG* from amino acids 1 to 735 (Table 3), and mutations outside of the S315 region have also been shown to be highly defective in INH activation. Sequencing only a fraction of the *katG* gene around the S315 region might leave mutations responsible for the drug resistance phenotype undiscovered. Nevertheless, in studies where the *katG* and *inhA* genes and their regulatory regions are entirely sequenced, the mechanism of resistance is not identified in up to 5% of the INH-resistant clinical isolates.

Additional mechanisms of resistance likely remain to be identified, and a worthwhile endeavor may be to revisit the early studies on INH mechanisms of action to determine other factors of INH and ETH resistance in *M. tuberculosis.* Knowledge of resistance genes and mutations has provided the means to develop GenoType MTBDR*plus*, a rapid nucleic acid–based test for assessing INH and RIF resistance (128). Moreover, it may provide strategies to develop new drugs that bypass the known mechanisms of drug resistance. For example, the natural product pyridomycin inhibits InhA without requiring KatG activation and is therefore active against highly INH-resistant *M. tuberculosis* clinical isolates carrying a *katG*

mutation (129). The identification of additional genes contributing to INH and ETH resistance will also expand our understanding of the mechanisms of drug action. Finally, in addition to specific mutations that confer resistance to every cell in a population, new studies that reveal the way in which a cell can become transiently phenotypically resistant to INH or ETH will be important in developing better ways to kill *M. tuberculosis* with INH and ETH and shorten chemotherapy.

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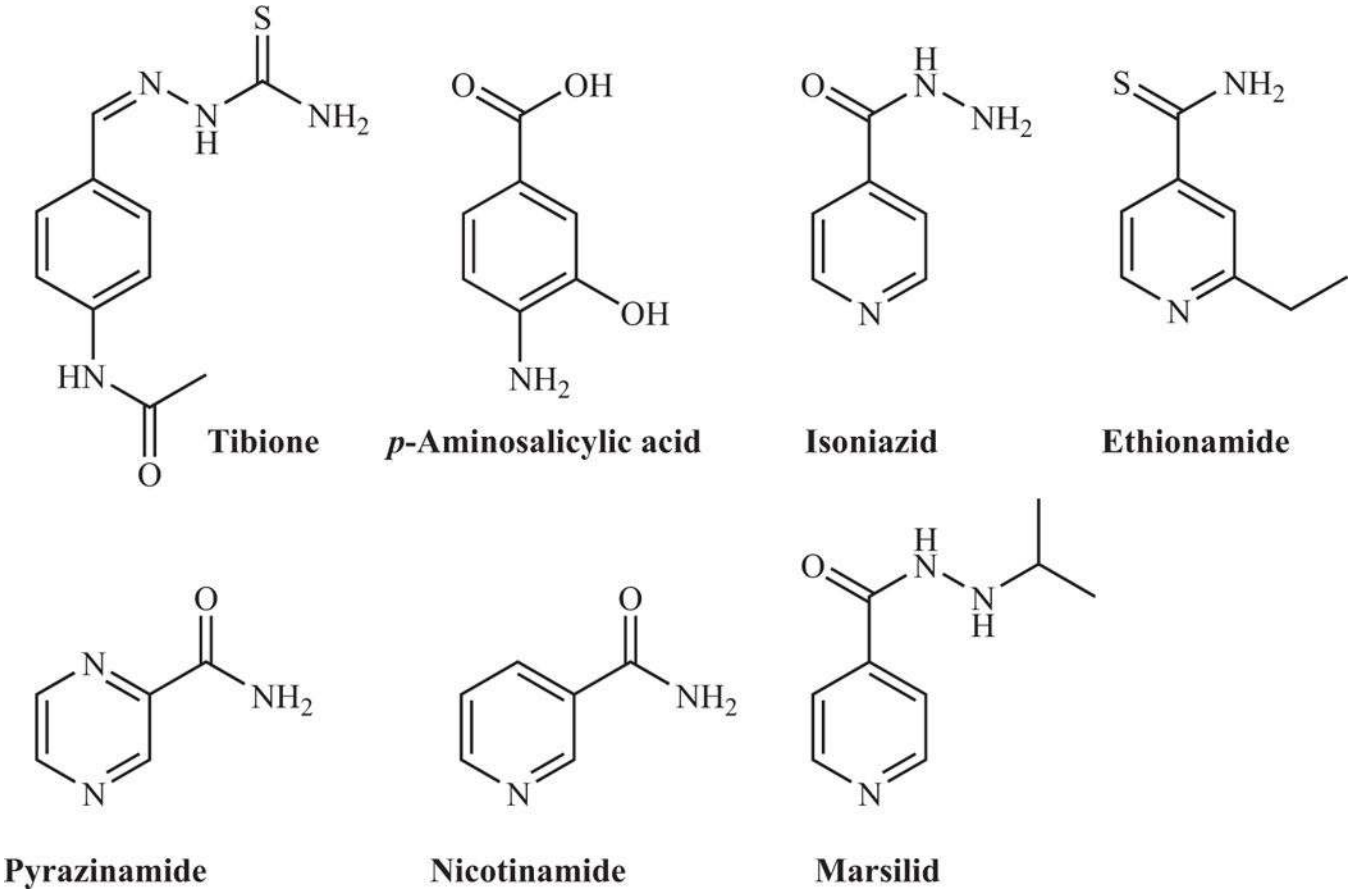

FIGURE 1.
Early synthetic antituberculosis drugs. doi:10.1128/microbiolspec.MGM2-0014-2013.f1

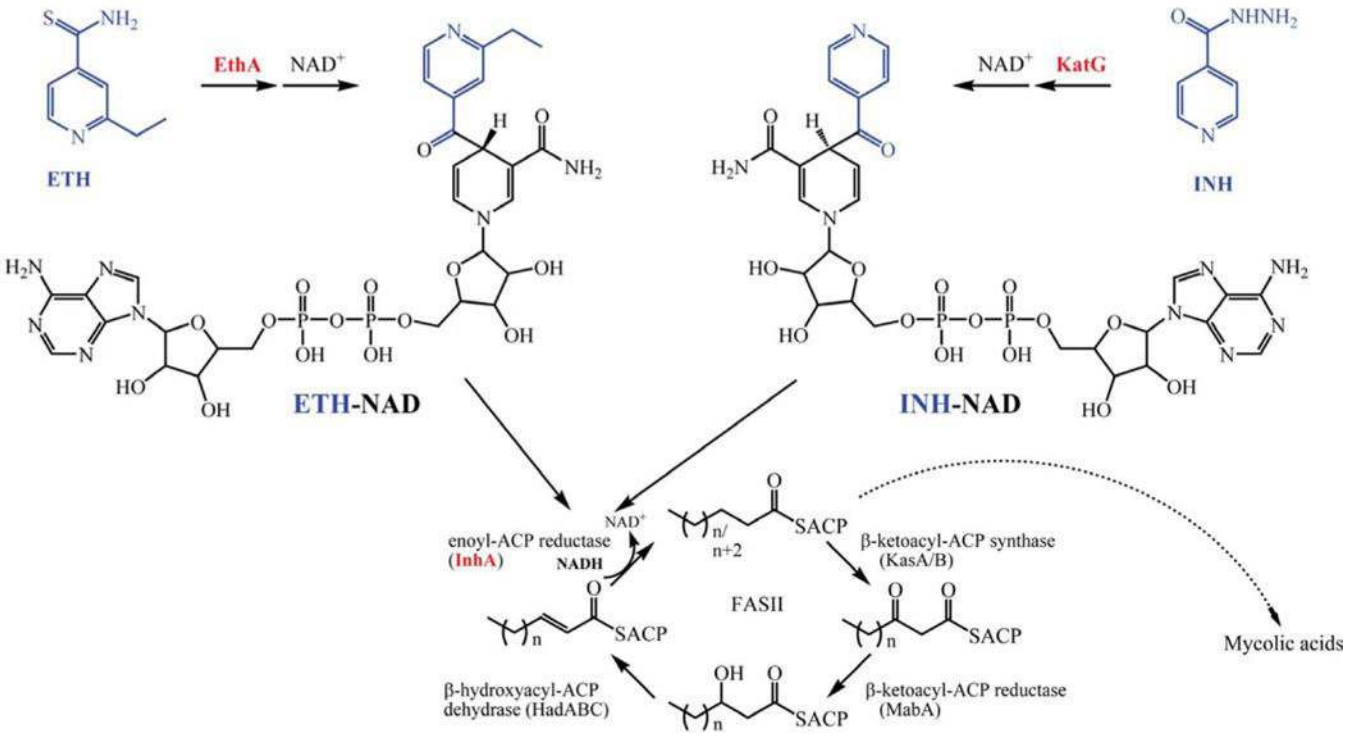

FIGURE 2.

Mechanism of action of INH and ETH. INH and ETH are activated by the catalase peroxidase KatG and monooxygenase EthA, respectively, to form a reactive species that binds to NAD^+. The resulting adducts, INH-NAD or ETH-NAD, inhibit the enoyl-ACP reductase InhA of the FASII system, resulting in mycolic acid biosynthesis inhibition. doi: 10.1128/microbiolspec.MGM2-0014-2013.f2

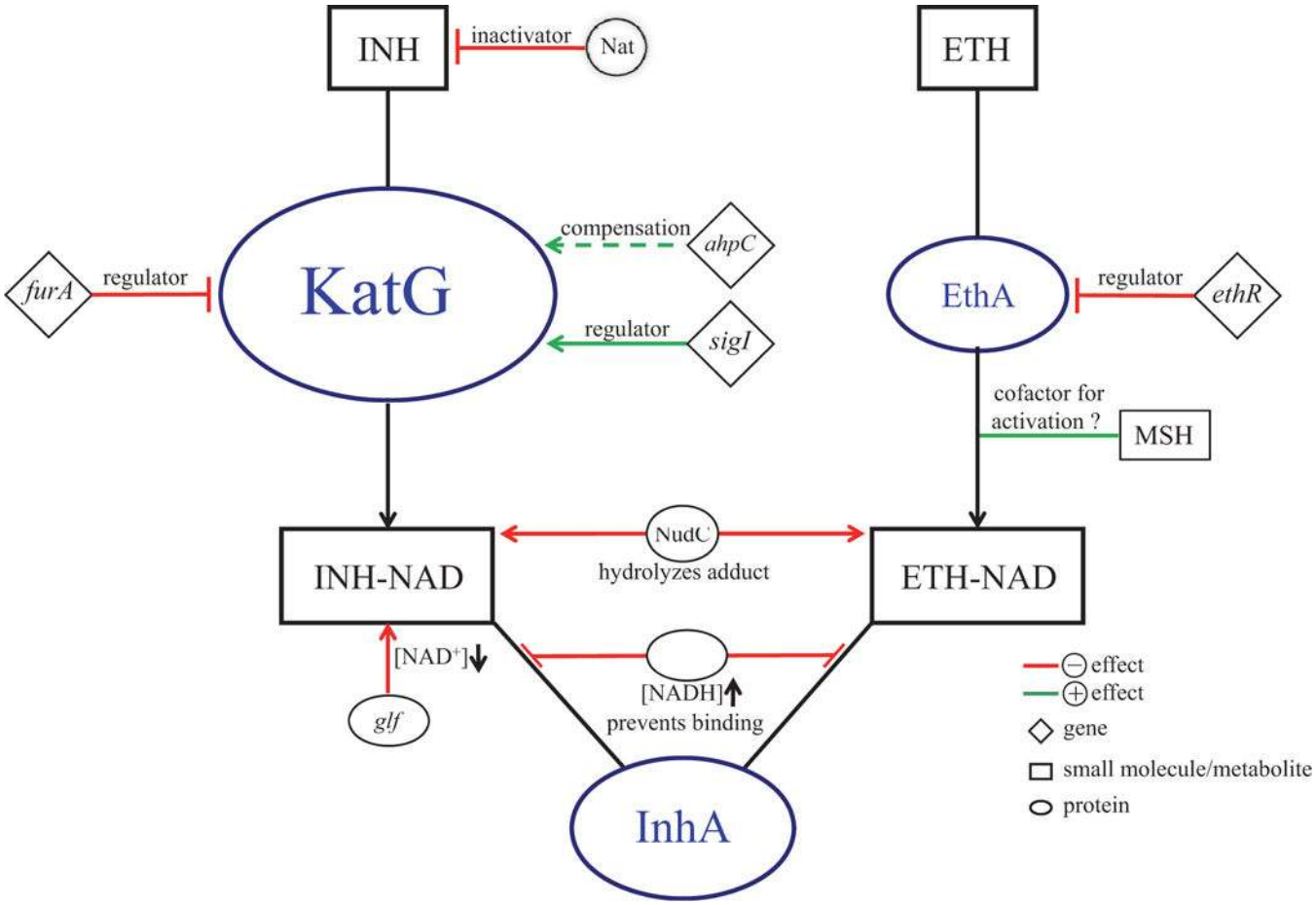

FIGURE 3.

Relationship among the genes and proteins involved in the resistance to INH and ETH in *M. tuberculosis*. Connections in red indicate a negative relationship (degradation of an active molecule, negative regulator of an enzyme) that would lead to resistance to INH and/or ETH; in green are positive actions that would increase a strain fitness or susceptibility to the drugs. The dashed line points to an interaction that does not result directly in INH resistance or susceptibility. doi:10.1128/microbiolspec.MGM2-0014-2013.f3

TABLE 1

Identified mutations in genes other than *katG* in INH-resistant *M. tuberculosis* strains[a]

***oxyR'-ahpC* intergenic region**	***furA-katG* intergenic region**	***Ndh***
t-89a (130)	c-1 ins (102)	R13C (67)
g-88a (130)	g-7a (96)	V18A* (66–67)
c-81t (131)	a-10c (96)	T110A (68)
g-74a (132)	g-12a (96)	R268H (66, 68)
c-72t (131)		G313R* (116)
g-67a (66)	***fabG-inhA* regulatory region**	
g-66a (133)	g-147t (88)	***iniB***
atgt-54 ins (92)	a-113c (134)	t198ins (135)
c-54t (132)	g-102a (134)	a211del (135)
c-52t (136)	a-92t (69)	222 12bp del (88)
g-51a (131)	g-67c (137)	N88S* (116)
t-49g (137)	g-47c,a (134, 138)	G192* (135)
g-48a (135, 139)	c-34t (138)	H481Q* (116)
g-46a* (102, 140)	t-24g (141)	
g-46 del* (66)	g-22c (142)	***iniA***
c-45t (84)	g-17t* (66, 135, 139, 143)	P3A (88)
t-44a (99)	a-16g (84)	nt282–286 del (88)
t-40c (144)	c-15t (66, 88, 135, 139, 140, 143)	H481Q* (88, 135)
c-39t (66, 135, 139, 140, 144)	t-12a (145)	R537H (88, 135)
t-34c,a (102, 140)	a-11t (66)	
t34 del (102)	t-8a*,g,c (66, 88, 140–142)	***iniC***
g-32a (140)	t-5a (146)	t79ins (135)
c-30t (66, 144)	c-4a (140)	a98ins (135)
c-20t* (102)	A5P (137)	W83G (88)
c-15t (66, 135, 139, 144)	V14L (137)	
c-12t (66, 135, 139, 140)	T21A (66)	***Rv0340***
c-10a,g,t (66, 135, 139, 140)		T143* (135)
g-9a* (66, 135, 139, 140, 144)	***inhA* ORF**	G149* (135)

g-6a (66, 140, 144)	M1L (147)	V163I (88, 135)
a-4g (140)	K8N (148)	
	I16T (149)	**nat**
***ahpC* ORF**	I21T,V (66, 88, 135, 139, 149)	G67R* (88)
P2S (69)	I25T (136)	Y177H (150)
L3K (140)	I47T* (66,149)	G207R* (88, 103)
L4R (130)	V78A (149)	
T5I (66)	S94A (59, 66,144)	***Rv1592c***
F10I (144)	I95P (151)	P42L (88)
D33N (84)	A190S (152)	V430A (88)
D73H* (66)	I194T* (66, 88)	
E76K (153)	R202G (133)	**fadE24**
L191K (66)	E217D (133)	-64 2 bp ins (88)
	T241M (142)	a-23c* (88)
oxyR'	T253A* (152)	
G12a (130)	D256N (152)	***Rv1772***
g18a (69)	I258T,V (135, 152)	T4A (88)
g27t (69)	Y259H (152)	
c28a (69)		***efpA***
c37t (99)	**kasA**	T15R* (116)
bp67 ins ggcg (99)	D66N* (66, 109)	I73T* (135)
g325t (99)	M77I* (88, 135)	Q513R (116)
a331c (99)	R121K (69) L245R (135)	E520V (135)
furA	G269S* (66, 69, 88, 109, 135,139)	***fabD***
S5P (88, 135)	G312S* (66, 69, 109, 135)	S275N* (88)
c34 del (96)	S341* (135)	A199T* (88)
A14V (96)	G387D (69)	
A46V* (116)	F413L (109)	***accD6***
L68F (94)		D229G* (88)
C97Y (94)	***srmR* homolog**	
	D3G (88)	***fbpC***
	M323T* (88)	G158S* (88)

[a] Asterisk (*), found in INH-resistant and/or INH-sensitive strains.

TABLE 2

Identified mutations in ETH-resistant *M. tuberculosis* strains[a]

ethA	***ethA***	***ethA***
M1R (62)	A234D (154)	C403G (62)
I9T (154)	t703 del (61)	R404L (154)
G11A (62)	Q246STOP (61)	G413D (61)
g32 del (154)	A248D (154)	c1254 del (154)
A20 ins (62)	Y250ST0P (154)	g1268 del (154)
a65 del (46)	cg754 ins (62)	c1290 del (61, 154)
H22P (62)	Q254P (154)	gc1322,1323 del (61)
Y32D (154)	Q254ST0P (154)	T453I (154)
a110 del (61, 154)	g768 del (61)*	Y461H (62)
G43S (61), C (46)	S266R (62)	R463D (61)
T44N (154)	Q269ST0P (62)	a1391 ins (62)
D49A (155)	Q271ST0P (154)	
Y50C (154)	L272P (62)	***fabG-inhA* regulatory region**
P51L (46)	P288R (154)	g-17t (61)
D55A (61)	Q291ST0P (154)	c-15t (38, 61, 156)
D58A (46)	R292ST0P (154)	
T61M (62)	C294F (154)	***inhA* ORF**
Y84D (46)	F302L (154)	I21T(61)
1bp271 del (46)	T324 ins (154)	S94A (61)
cg282–283 del (154)	L328M (155)	I95P (151)
g337 del (154)	S329L (62)	
a338 ins (61)	L333R (155)	*mshA*
a342 del (154)	I338S (61)*	N111S* (62, 70)
G124D (62)	T342K (46)	Q128STOP[a](70)
G139S (154)	d1029 del (154)	V171G(62)
Y140 STOP (154)	N345K (155) (154)	A187V(62)
Q165P (62)	A352P (154)	R273C[b] (70)

W167STOP (154)	g1054 del (154)	G299C[b] (70)
T186K (46)	P378L (154)	Q331STOP[b] (70)
g593 del (154)	A381P (46)	G356D[b] (Z0)
c613 del (155)	t1152 del (154)	E361A[b] (70)
gt638–639 del (155)	G385D (61)	
D219G (154)	Y386C (62)	*ethR*
E223K (61)	S390F (154)	A95T (62)
g673 ins (154)	W391STOP (154)	F110L (62)
tc675 ins (62)	T392A (61)	
T232A (155)	L397R (155)	

[a] Asterisk (*), also found in ETH-sensitive strains.

[b] Mutations found in *in vitro* cultures.

TABLE 3

Identified *katG* mutations in INH-resistant *M. tuberculosis* strains[a]

Complete deletion (30, 88, 134, 141, 157)	E217G,del (84, 158)	W397Y (88) STOP (66)
Partial deletion (138, 159–161)	N218K (153)	A409D,R,T,V (142,152, 153, 162)
V1A (84) L (92)	Q224E (163)	Y413H (133)
P2S (163)	Y229F (164)	K414N (165)
a17 ins (139)	V230A (153)	R418Q (166)
c30 del (160)	P232R,S (139, 167)	D419A,Y,E, H (59,139)
T11A (160)	G234E (102) R (153)	M420T (167)
T12P (160)	N236T (153)	A424E V (163)
S17N (163)	A243S (163)	G428R (168)
G19D (163)	A245V* (146)	P429S (163)
a98ins (160)	R249C (159)	c1297 ins, c1305 del (167)
N35D (160)	T251M (92)	Q434stop (88)
g109del (160)	F252L (84)	t1311 ins (139)
W38stop (66)	R254L (139)	W438R (88)
L48Q (146)	M257I,T (143, 146)	a1329 ins (139)
a149 del (102)	N258S (163)	c1339 del (139)
A61T (160)	E261Q (142)	L449F (169)
c185 ins (160)	T262R (84, 141)	S457I (165)
D63E (88, 141)	A264T,V* (152,163)	K459ST0P (168)
A65T (163)	H270Q (170)	R463L* (85, 141, 162,171)
A65 or cccc ins (84,157)	T271P (172)	W477stop (84)
A66P (163)	G273C,S (139, 173)	R484S (102)
I71N (84)	T275A,P, (84, 159, 162,171)	G485V (84)
D72G (153) K (66)	G279D, A* (59, 139)	K488N (157)
D73N (160)	P280H,L*,P (92, 93,152)	R489S (167)
D74Y, G (153)	A281V (163)	G490S (102, 139)
M84I (153)	G285C,D,V (116, 145, 152)	G491C (135, 143)
T85P (92)	E289D (153)	N493H (142)
Q88R (157)	A291P,V (152, 174)	G494D (59)

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W90R (139) STOP (88, 135, 163)
W91R (88)
D94A (84)
G96C (153)
H97R (139)
G99E (84)
R104L (141), Q (92)
M105I (93)
A106V (92)
W107R (92)
W107ST0P (102)
H108E,Q (84, 141)
A109V (139)
A110V (59, 84)
D117A (143)
G120del (84)
G121C (92) V (157)
A122del (84)
G123E (167)
g371 del (167)
G125C (153)
H125 ins (171)
M126I (163)
Q127E,P (153, 167)
R128P (66) E (174)
F129S (116)
N133T (167)
N138S,H*,T (61, 66, 84, 88, 178)
A139P (84)
S140A,N (84)
L141F (59)
D142A (84)
L293V (139)
Q295K,P,STOP (130, 146, 157)
M296V (145)
Q297V (146)
G299S (139) C (153) A (133)
W300D,G,C,ST0P (69, 84, 138, 157)
gc900 ins (138)
S302R (139, 163)
Y304S (165)
T308P (176)
G309C,S,V (177–179)
D311E (180) G (153) Y (179)
A312G,V (152)
S315T,N,I,R,G,L (59, 66, 84, 88, 135, 139, 141–144, 157)
G316D, S* (66)
I317L (153)
E318G,V (152, 180)
W321G (66) R (153)
T322A,I (136, 179)
T324A,P (146, 179)
T326D (181)
K327I (178)
W328L,C,F,S,G (84, 155, 172,182,183)
D329A,C (138, 145)
S331C (180)
I334T (84)
I335T (84, 141) V (184)
L336R,P (88)
Y337F,C (163)
W341S,G (59,139)
T344A,P (152,175)
K345T (92)
R496L (168)
P501A (157)
W505S (175), R (163)
D511del (84)
D513del (84)
R515C (66, 84,135)
L521del (84)
Q525P (84)
N529D (88)
D542H (167)
L546P (172)
A550D (163)
E553K (59)
c1667del (66)
F567S (84)
D573N (92)
A574V (84) E (66)
L587I,M,P* (66, 84)
P589T (163)
G593D (84)
R595stop (59)
E607K (92)
M609I (163)
L617del (84)
ac1849 ins (157)
L619P (84)
G629S (88, 141)
R632C (167)
L634F (84)
A636E (92)
L653P (92)
F658V (59)

K143T (92)	P347L* (152)	L662R (139)
A144V (139)	A350S,T (84, 141)	G685R (135)
R146W (93)	Q352ST0P (155)	D695A,G (162,172)
L148A,P (84, 170)	A361D (133)	G699Q (102)
Y155C (92) S (157)	T363A (152)	S700P (84,157)
Y155C (92) S (157)	T363A (152)	S700P (84, 157)
S160L (84)	P365S* (152)	V708P (146)
cc478–479 del (167)	F368L* (152)	V710A (84)
A162T (92)	G372 ins (88)	c139 del (139)
G169A (163)	S374P* (152)	A713P (66)
A172T (66, 84) V (153)	L378P (133)	A714P (84)
M176I,T (153, 167)	A379T (142) V (133)	A716P (141)
T180C (84) K (66)	T380I (153)	Q717P (66)
G186V,H (153)	D381G (141)	V725A (146)
W191R, STOP (102, 172)	S383P (167)	A726T (139)
g572 del (153)	L384R (92)	A727D (159)
WE191–192 del (167)	D387H (167)	W728C (92)
E195K (61)	P388L,S (152)	D735 del (92)
W198stop (84)	T390I (153)	D735A (66, 84), N (92)
K200E (139), STOP (84)	L390 ins (146)	
W204R	I393N (84)	

[a] Asterisk (*), found in INH-resistant and/or INH-sensitive strains.

TABLE 4

Biochemical activity of KatG variants (87, 146, 167)

	Variant		
Activity	**Similar to wt**	**Partial**	**None**
Catalase peroxidase	A110V, A139P, A245V, S315N, S315T, R463L, L587M, L619P, L634F, D735A	L48Q	L141F, T275P, Q295P, G297V, T324P, L587P
INH oxidase	L48Q, A110V, A245V, R463L	A139P, Q127E, N133T, L141F, P232S, Q295P, G297V, T324P, S383P, D387H, D419H, M420T, R489S, L634F, D735A	M176T, S315 (N,R,T), D542H, L619P, R632C