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Molecular Investigation of Resistance to the Antituberculous Drug Ethionamide in Multidrug-Resistant Clinical Isolates of *Mycobacterium tuberculosis*▽

F. Brossier,[1,2] N. Veziris,[1,2] C. Truffot-Pernot,[2] V. Jarlier,[1,2] and W. Sougakoff[1,2,3]*

National Reference Centre for Mycobacteria, Hôpital Pitié-Salpêtrière, AP-HP, Paris, France[1]; Université Pierre et Marie Curie (UPMC), Université Paris 06, EA1541, and Faculté de Médecine Pitié-Salpêtrière, Paris, France[2]; and INSERM, UMRS 872-12 (Pitié-Salpêtrière), Laboratoire de Recherche Moléculaire sur les Antibiotiques (LRMA), Paris, France[3]

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Ethionamide (ETH) needs to be activated by the mono-oxygenase EthA, which is regulated by EthR, in order to be active against *Mycobacterium tuberculosis*. The activated drug targets the enzyme InhA, which is involved in cell wall biosynthesis. Resistance to ETH has been reported to result from various mechanisms, including mutations altering EthA/EthR, InhA and its promoter, the NADH dehydrogenase encoded by *ndh*, and the MshA enzyme, involved in mycothiol biosynthesis. We searched for such mutations in 87 clinical isolates: 47 ETH-resistant (ETH^r) isolates, 24 ETH-susceptible (ETH^s) isolates, and 16 isolates susceptible to ETH but displaying an intermediate proportion of resistant cells (ETH^{Sip}; defined as ≥1% but <10% resistant cells). In 81% (38/47) of the ETH^r isolates, we found mutations in *ethA*, *ethR*, or *inhA* or its promoter, which mostly corresponded to new alterations in *ethA* and *ethR*. The 9 ETH^r isolates without a mutation in these three genes (9/47, 19%) had no mutation in *ndh*, and a single isolate had a mutation in *mshA*. Of the 16 ETH^{Sip} isolates, 7 had a mutation in *ethA*, 8 had no detectable mutation, and 1 had a mutation in *mshA*. Finally, of the 24 ETH^s isolates, 23 had no mutation in the studied genes and 1 displayed a yet unknown mutation in the *inhA* promoter. Globally, the mechanism of resistance to ETH remained unknown for 19% of the ETH^r isolates, highlighting the complexity of the mechanisms of ETH resistance in *M. tuberculosis*.

Ethionamide (ETH), a second-line antituberculous drug, is a structural thioamide analogue of isoniazid (INH), the cornerstone of front-line tuberculosis (TB) treatment. ETH is considered to be the most active antituberculous drug after aminoglycosides and fluoroquinolones and is a component of most of the drug regimens used for treating multidrug-resistant (MDR) TB (MDR-TB) or suspected MDR-TB (25, 32). To date, drug susceptibility testing (DST) for ETH relies mainly on phenotypic tests because the molecular mechanisms of ETH resistance are not fully understood. *In vitro* phenotypic investigation of ETH resistance is experimentally difficult and can yield discordant results according to the experimental method and resistance breakpoint values used, which still remain matters of debate (26). Therefore, a genotypic approach would be of great value to improve and hasten DST and the management of MDR-TB, as well as reduce the proportion of patients inadequately treated on the basis of erroneous DST results, consequently limiting the emergence of extensively drug-resistant tuberculosis among patients treated for MDR-TB.

ETH and INH share the same molecular target, the NADH-dependent enoyl-acyl carrier protein reductase InhA of the fatty acid biosynthesis type II system, which is involved in the synthesis of mycolic acids (2, 19). Consequently, cross-resistance to these two antibiotics can be observed in clinical isolates. However, though ETH is a structural analogue of INH, cross-resistance between ETH and INH does not occur systematically. Strains with low-level resistance to INH frequently display low-level ETH resistance, whereas high-level INH-resistant (INH^r) strains typically remain ETH susceptible (ETH^s) (6, 7, 21). This apparent paradox is due to the fact that INH and ETH are prodrugs that need to be activated by mycobacterial enzymes in order to exert their antimicrobial activity, with each drug being activated by a specific mechanism. INH is activated by the *katG*-encoded catalase-peroxidase (33), whereas ETH is activated by the *ethA*-encoded NADPH-specific FAD-containing mono-oxygenase (3, 10, 27). In addition, the expression of EthA is negatively regulated by EthR, a regulator that interacts directly with the *ethA* promoter region (11).

Resistance to ETH or INH has previously been reported to result primarily from (i) mutations altering the activator proteins KatG (14, 23) and EthA (3, 10, 21), leading to resistance to INH and ETH, respectively; (ii) mutations in the InhA protein targeted by INH and ETH, which prevents the corresponding activated forms of the drugs from binding the target, leading to cross-resistance to both antibiotics (2, 29); (iii) mutations in the *inhA* promoter region that cause overexpression of the target InhA and cross-resistance to the two drugs (3, 10, 16); and (iv) mutations in the negative transcriptional regulator EthR, specifically leading to ETH resistance (ETH^r) (3). A higher prevalence of mutations in *katG* (associated with a high level of INH resistance) than in the *inhA*/*inhA* promoter region (associated with a low level of INH resistance and coresistance to ETH) accounts for the fact that cross-resistance to the two

* Corresponding author. Mailing address: Laboratoire de Bactériologie-Hygiène, LRMA, UMRS 872-12 site Pitié-Salpêtrière, 91 boulevard de l'Hôpital, F-75634 Paris Cedex 13, France. Phone: (33) 1 40 77 97 46. Fax: (33) 1 45 82 75 77. E-mail: wladimir.sougakoff@crc.jussieu.fr.

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drugs is not systematic in clinical strains, making the use of ETH in cases of INH resistance possible (30). Finally, some INH- and ETH-resistant clinical isolates of *Mycobacterium tuberculosis* have been reported to have no mutations in the genes known to be involved in INH or ETH resistance (i.e., in *katG*, *inhA*, *ethA*, and *ethR*) (4, 5, 14, 21) but display mutations in other genes possibly involved in INH/ETH resistance, such as *ndh*, *mshA*, and *dfrA*. *ndh* encodes an NADH dehydrogenase that regulates the NADH/NAD^+ ratio. Mutations in *ndh* would result in an increased intracellular NADH concentration and, concomitantly, coresistance to INH and ETH by competitively inhibiting the binding of INH-NAD and ETH-NAD adducts to InhA (20, 30). Such mutations have been demonstrated to cause INH and ETH resistance in *Mycobacterium smegmatis* (20) and *Mycobacterium bovis* BCG (30) but not yet in *M. tuberculosis* (the *ndh* mutations reported to date in *M. tuberculosis* have also been observed in INH-susceptible [INH^s] isolates) (4, 9, 13, 18, 23). *mshA* encodes a glycosyltransferase, an enzyme involved in mycothiol biosynthesis, a major low-molecular-weight thiol in *M. tuberculosis*. Mycothiol would promote ETH activation via the mono-oxygenase EthA. Mutations in *mshA* have been proposed to cause a defect in ETH activation but have never been documented in clinical isolates and have been found only in *in vitro*-selected INH- and ETH-resistant *M. tuberculosis* mutants (28). Finally, *dfrA* encodes dihydrofolate reductase (DHFR), an enzyme essential for nucleic acid synthesis. Recently, DHFR was proposed to be a possible target of INH because one of the isonicotinoyl adducts generated by the activation of INH by KatG has been shown to inhibit the *M. tuberculosis* DHFR (1). However, the importance of *dfrA* in INH and ETH resistance is uncertain, as a recent investigation of *dfrA* from INH^r clinical isolates found no *dfrA* mutation in 127 INH^r strains (15).

Few systematic studies on the distribution of nucleotide changes affecting the above-mentioned genes and focused primarily on a limited number of genes have been conducted (4, 10, 13, 17, 21, 24). These studies also included a limited number of ETH^r strains, usually less than 25 (4, 10, 13, 21, 24). In the present study, we investigated a set of 87 clinical isolates showing different patterns of susceptibility to ETH, including 47 exhibiting ETH resistance and 16 displaying ETH susceptibility with an intermediate proportion of resistant cells (ETH^{Sip}), for the presence of mutations in *ethA*, *ethR*, *katG*, *inhA* and the *inhA* promoter region, *mshA*, *ndh*, and *dfrA*.

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MATERIALS AND METHODS

Drug susceptibility testing and strains. DST was performed on Lowenstein-Jensen medium using the standard proportions method (8) and concentrations of 20 mg/liter for ETH and 0.1, 0.2, 1, and 10 mg/liter for INH (31). ETH^r was defined, as recommended, as ≥10% resistant mutants at 20 mg/liter (31). For the purpose of this study, we distinguished a subgroup of isolates exhibiting an abnormal proportion of ETH-resistant cells (≥1%) that was still too low (<10%) to meet the WHO definition of resistance. This particular pattern was referred to as "susceptible but intermediate proportion" (Sip), i.e., ≥1% but <10% resistant mutants. Resistance to INH was defined as ≥1% resistant mutants at 0.2 mg/liter. Two levels of resistance were distinguished: a low level of resistance (LL), defined as resistance to INH at concentrations of ≥0.2 mg/liter but <1 mg/liter, and a high level (HL) of resistance, defined as resistance to INH at concentrations of ≥1 mg/liter.

A total of 87 *M. tuberculosis* complex isolates from TB cases diagnosed in France and received at the French National Reference Center for Mycobacteria (mainly in the framework of MDR national surveillance) were collected over a 1-year period. These isolates corresponded to all of the INH^r isolates ($n = 63$) received and a random selection of susceptible isolates ($n = 24$). On the basis of the results of DST, the 87 isolates were classified as ETH^r ($n = 47$, all of which were resistant to INH and including 39 MDR isolates), ETH^s ($n = 24$, 13 of which were resistant to INH and including 11 MDR isolates), and ETH^{Sip} ($n = 16$, all of which were resistant to INH and including 13 MDR isolates).

DNA sequencing of genes associated with drug resistance. For all isolates, the entire *ethA*, *ethR*, *katG*, and *inhA* genes and the *fabG1-inhA* regulatory region (up to position −189 upstream from the *fabG1* gene) were sequenced. For isolates displaying wild-type *ethA*/*ethR*, *inhA*, and *inhA* promoter, the entire *ndh*, *dfrA*, and *mshA* genes were sequenced. Genomic DNA was isolated from bacteria grown on Lowenstein-Jensen medium. A loop of culture was resuspended in water (500 μl) and killed by heating at 95°C for 15 min.

DNA (5 μl) obtained by heat shock extraction (1 min at 95°C and 1 min in ice, repeated five times) was submitted to PCR amplification using the following protocol: an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at the primer-dependent annealing temperature (T_a), and 1 min at 72°C, ending with a final extension step of 7 min at 72°C. *ethA*, *ethR* and the intergenic region, *dfrA*, *ndh*, and *mshA* were amplified and sequenced using the primers in Table 1. After amplification, unincorporated nucleotides and primers were removed by filtration with Microcon 100 microconcentrators (Amicon Inc., Beverly, MA), and the amplicons were sequenced using a BigDye Terminator cycle sequencing ready kit (Applied Biosystems, Courtaboeuf, France).

Statistical analysis. The 47 ETH^r, 16 ETH^{Sip}, and 24 ETH^s isolates were compared for mutations using Fisher's exact test. *P* values were two-tailed, and *P* values of ≤0.05 were considered significant.

Nucleotide sequence accession numbers. The nucleotide sequences determined for the mutant genes were deposited in the GenBank database under the following accession numbers: HM587450 to HM587471 for the *ethA* mutants, HM587472 and HM587473 for the *ethR* mutants, and HM587474 and HM587475 for the *mshA* mutants.

RESULTS

Of the 47 ETH^r/INH^r isolates, 22 were mutated in *ethA*. The difference in the proportion of ETH^r isolates with a mutation was significant compared to differences in proportions for the ETH^s and ETH^{Sip} isolates ($P = 2 \times 10^{-10}$ and $P = 0.009$, respectively). Ten of the 22 *ethA* mutants had at least one missense mutation ($n = 8$ with single mutations, $n = 2$ with double mutations), 7 had an AMB mutation, and 5 had an insertion of one to three nucleotides in *ethA*. Nearly all of these mutations (16/17) represented new mutations in EthA (Table 2), and only 1 (S266R) had been previously reported (4). Twelve of these 22 isolates also possessed a mutation in the *inhA* promoter region, and 1 had both a mutation in the *inhA* promoter region and a new mutation in EthR (F110L). Seven isolates showed a remarkable combination of mutations: Q269AMB in EthA combined with −15C → T in the *inhA* promoter region and A110V in KatG.

The 25 remaining ETH^r isolates had a wild-type *ethA* (Table 2). Among these isolates, nine displayed a mutation in the *inhA* promoter region only, whereas four others possessed the mutation plus another mutation in either *inhA* (S94A; $n = 3$) or EthR (new mutation A95T; $n = 1$). Three other isolates had a mutation (S94A) in *inhA* only. Finally, the nine remaining ETH^r isolates with a wild-type *ethA* had no mutation in EthR, InhA, or the *inhA* promoter. *dfrA*, *ndh*, and *mshA* were investigated in the nine isolates, and only one was found to have a mutation in one of these genes (double mutation V171G and A187V in *mshA*). Finally, investigating the mechanisms of INH resistance in the 47 ETH^r isolates showed the presence of

TABLE 1. Oligonucleotide primers used in PCR and DNA sequencing

Resistance gene	Primer	Sequence (5′ → 3′)	Accession no.	Hybridization temp (°C)	PCR product size (bp)	Reference or source
ethA (1,470 bp)	ETHA1	ATC ATC GTC GTC TGA CTA TGG	BX842584	64	1,278	21
	ETHA9	CCT CGA GTA CGT CAA GAG CAC				
	ETHA10	CGT TGA CGG CCT CGA CAT TAC		64	342	21
	ETHA8	GGT GGA ACC GGA TAT GCC TG				
ethR (651 bp)	ETHR1	CGC TGA CAC CGG AGA TTC C	BX842584	59	915	This study
	ETHR4	CGC TCC TAT ATG ACC GCA CG				
ndh (1,392 bp)	Ndh1s	ACT GAG TAC CTG GCA GGC TG	BX842578	57	1,479	This study
	Ndh4as	GCT AAC TGA ACT CGC TCA TCG				
mshA (1,443 bp)	mshA5s	GCG TGT CAC TTC GGT TCC TGC	BX842573	57	749	This study
	mshA6as	CAC CGG GAT GGA CCA CGT CG				
	mshA11s	GGC CGC CGT GAA GAA CGC GG		62	1,020	This study
	mshA12as	CAG CTC GAC GAT CAG CGC GG				
dfrA (480 bp)	dfr2s	CGC TTG CGG GGG ACG AAG C	BX842580	61	480	This study
	dfr4as	GCA TCC GGC GAT CAA AGC TCC				
katG (2,223 bp)	katA	CCC GAT AAC ACC TCC TG	BX842578	59	817	5
	katCas	GTT TCG ACG TCG TTC ATG GC				
	kat2	CTC GGC GAT GAG CGT TAC AG		59	1,318	5
	kat4as	CCA GCG GTA AGC GCT TGT AG				
	katC	CCG AGT ACA TGC TGC TCG AC		59	609	5
	katEas	GGT GAT CGC ACA TCC AGC AC				
fabG1-inhA promoter	Pro1	TCA ATA CAC CCG CAG CCA	BX842576	53	493	5
	Pro2	GTC ATC CGC ATG AGG AAT				
inhA (810 bp)	INHA1	AGG ACG CAC ATG ACA AGC	BX842576	53	412	5
	INHA2	TCA TGA TCG GCA GCA GCG				
	INHA3	CCA CAT CTC GGC GTA TTC		53	601	5
	INHAD	CGA AAT GCA GGT AGT GCT C				

KatG mutations in 35 isolates (S315T in 26, A110V in 7, D419H in 1, and F658V in 1), the *inhA* promoter in 26 isolates, and/or InhA (S94A) in 6 isolates (see Table 2 for the details regarding mutation combinations).

The 16 ETH^{Sip} isolates showed fewer mutations than the ETH^{r} isolates ($P = 0.009$) and more mutations than the ETH^{s} isolates ($P = 0.004$). Among the 16 ETH^{Sip} isolates, 7 were mutated in *ethA* (44%), of which 3 had double mutations: V7A and S266R, L129R and P192T, or G124D and A199V. The isolate with the last mutation also had a −102G → A mutation in the *inhA* promoter region (Table 2). Most of these mutations (7/8) were new (Table 2). Mutations in codon 43 were also described in other studies: G43C by DeBarber et al. (10) and G43S by Morlock et al. (21). The other nine ETH^{Sip} isolates had no mutations in *ethA* or *ethR*, *inhA*, or the *inhA* promoter. One of these nine isolates had an N111S mutation in *mshA*, whereas the remaining eight had wild-type *dfrA*, *ndh*, and *mshA* sequences. Resistance to INH in the 16 ETH^{s} isolates was accounted for by mutations in KatG (S315T).

Finally, among the 24 ETH^{s} isolates, 23 had no mutations in *ethA*/*ethR*, *inhA*/*inhA* promoter region, *dfrA*, *ndh*, or *mshA*, whereas a single isolate displayed a rare mutation (−47G → C) with an unknown effect in the *inhA* promoter region. Resistance to INH in the 13 ETH^{s} isolates displaying INH resistance was accounted for by mutations in KatG: S315T ($n = 10$), R595OPA ($n = 1$), G494D ($n = 1$), or E553K ($n = 1$). No KatG mutation was found in the 11 ETH^{s}/INH^{s} isolates.

Overall, the analysis of the distribution of mutations in our collection of clinical isolates indicated that 81% had at least one mutation in one of the major genes involved in ETH resistance, and these were distributed as follows: 39% in *inhA* or the *inhA* promoter alone, 24% in *ethA* alone, 31% in the *inhA* promoter and *ethA*, 3% in the *inhA* promoter and *ethR*, and 3% in the *inhA* promoter, *inhA*, and *ethR*.

DISCUSSION

By studying a large number of clinical isolates, the four major genes clearly involved in ETH resistance (*ethA*/*ethR*, *inhA*/*inhA* promoter), and three genes proposed to be involved in this resistance (*mshA*, *ndh*, *dfrA*), we have highlighted the most relevant genetic patterns involved in ETH resistance in both ETH^{r} and ETH^{Sip} isolates. In 81% (38/47) of the ETH^{r} clinical *M. tuberculosis* isolates, ETH resistance could be attributed to mutations in at least one of the four major genes or regions known to be involved in ETH resistance (*ethA*, *ethR*, *inhA*, or the *inhA* promoter region). More specifically, 47% (22/47) of the ETH^{r} isolates had mutations in *ethA*, a finding that agrees with reports in three other publications that indicated the proportion of genes with mutations to range from 37% (15/41) to 100% (11/11) (4, 10, 21), with the overall proportion being 51% (39/76) when the results of the three studies are combined. In these previous publications, the mutations in *ethA* were nonsynonymous, affecting 22 distinct

TABLE 2. Sequencing results for *ethA*, *ethR*, *inhA* and its promoter region, *dfrA*, *ndh*, *mshA*, and *katG* in 87 isolates of *M. tuberculosis*

Resistance phenotype[a]		Sequencing result[b]								Resistance level (no. of isolates)
ETH	INH	EthA[c]	EthR	*inhA* promoter	InhA	DfrA	Ndh	MshA	KatG	
R	R	M1R (ATG → AGG)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		G11A (GGC → GCC)-S266R (AGC → AGG)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		T61M (ACG → ATG)	wt	wt	wt	ND	ND	ND	wt	LL (1)
		Q165P (CAG → CCG)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		CG insertion after 754	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		L272P (CTA → CCA)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		S329L (TCA → TTA)-C403G (TGT → GGT)	wt	wt	wt	ND	ND	ND	S315T	LL (1)
		Y386C (TAC → TGC)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		Y461H (TAC → CAC)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		Q269AMB (CAG → TAG)	wt	−15C → T	wt	ND	ND	ND	A110V	LL (6), HL (1)
		G124D (GGC → GAC)	wt	−15C → T, 102G → A	wt	ND	ND	ND	wt	HL (1)
		TC insertion after 675	wt	−15C → T	wt	ND	ND	ND	S315T	HL (1)
		A insertion after 1391	wt	−15C → T	wt	ND	ND	ND	S315T	HL (2)
		GGC insertion after 59	wt	−8T → C	wt	ND	ND	ND	S315T	HL (1)
		H22P (CAC → CCC)	F110L[c]	−15C → T	wt	ND	ND	ND	S315T	HL (1)
		wt	A95T[c]	−15C → T	wt	ND	ND	ND	wt	HL (1)
		wt	wt	−15C → T	wt	ND	ND	ND	S315T	HL (6)
		wt	wt	−15C → T	wt	ND	ND	ND	wt	LL (3)
		wt	wt	−15C → T	S94A	ND	ND	ND	wt	LL (3)
		wt	wt	wt	S94A	ND	ND	ND	wt	LL (2)
		wt	wt	wt	S94A	ND	ND	ND	D419H	LL (1)
		wt	wt	wt	wt	wt	wt	A187V-V171G[d]	S315T	HL (1)
		wt	wt	wt	wt	wt	wt	wt	S315T	HL (6)
		wt	wt	wt	wt	wt	wt	wt	F658V	LL (1)
		wt	wt	wt	wt	wt	wt	wt	wt	LL (1)
Sip	R	V7A (GTT → GCT)-S266R (AGC → AGG)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		S266R (AGC → AGG)	wt	wt	wt	ND	ND	ND	S315T	HL (2)
		G43V (GGC → GTC)	wt	wt	wt	ND	ND	ND	S315T	LL (1)
		F66L (TTC → CTC)	wt	wt	wt	ND	ND	ND	S315T	LL (1)
		L129R (CTC → CGC)-P192T (CCG → ACG)	wt	wt	wt	ND	ND	ND	S315T	HL (1)
		G124D (GGC → GAC)-A199V (GCC → GTC)	wt	−102G → A	wt	ND	ND	ND	S315T	HL (1)
		wt	wt	wt	wt	wt	wt	N111S[d]	S315T	HL (1)
		wt	wt	wt	wt	wt	wt	wt	S315T	HL (8)
S	R	wt	wt	wt	wt	wt	wt	wt	S315T	HL (9)
		wt	wt	−47 G → C	wt	wt	wt	wt	S315T	HL (1)
		wt	wt	wt	wt	wt	wt	wt	R595OPA	HL (1)
		wt	wt	wt	wt	wt	wt	wt	G494D	HL (1)
		wt	wt	wt	wt	wt	wt	wt	E553K	LL (1)
S	S	wt	wt	wt	wt	wt	wt	wt	wt	S (11)

[a] R, resistant; Sip, susceptible but intermediate proportion; S, susceptible.
[b] Amino acid (one-letter code) mutations are generally given for all genes except the *inhA* promoter region, and the corresponding nucleotide changes are given in parentheses. The position of the nucleotide insertions in *ethA* is indicated. The new mutations found in EthA and EthR are underlined. wt, wild type; ND, not done.
[c] New mutations: F110L (TTC → CTC) and A95T (GCC → ACC).
[d] New mutations: A187V (GCA → GTA), V171G (GTG → GGG), and N111S (AAC → AGC).

codons, and the other mutations were either deletions ($n = 8$) or insertions ($n = 2$). In our study, the alterations detected in *ethA* were also scattered throughout the gene and consisted of missense mutations, nonsense mutations, and insertions. Most of the mutations identified in our study were new and affected codons different from those cited in the three previous reports. Overall, these data clearly confirm a high degree of diversity in the mutations affecting *ethA*, as previously observed in other mycobacterial resistance genes, such as *katG* and *pncA*, in which hundreds of distinct mutations have been described thus far (5, 22). However, the S315T mutation is by far the most frequent KatG mutation observed in INH^r clinical isolates, which is in contrast to the lack of a prominent mutation in EthA. The predominance of the codon 315 mutation in KatG has been explained by the need for *M. tuberculosis* to maintain sufficient catalase-peroxidase activity to protect against oxidizing species produced by neutrophils of the infected host. No predominant *ethA* mutations have emerged in the ETH^r clinical isolates investigated thus far, suggesting the existence of another enzyme with redundant functions. The genome of *M. tuberculosis* possibly encodes more than 30 mono-oxygenases (3), and the function of EthA, as opposed to that of KatG, does not seem to be essential in *M. tuberculosis*.

In the present study, we found two ETH^r isolates with two different mutations in EthR, F110L and A95T. These two mutations are, to the best of our knowledge, the first to be described in *ethR* in relation to ETH resistance. The crystal structure of EthR, which negatively regulates the expression of EthA by interacting directly with the *ethA* promoter region, was previously reported to be a homodimer containing a clas-

sical DNA binding domain (helix-turn-helix motif) and ligand-binding site made of two helices (α4 to α9) which is required to control the conformational change that prevents the binding of EthR to its target DNA (11, 12). On the basis of this crystal structure, F110 is localized in helix α5, with its aromatic side chain oriented in the hydrophobic tunnel constituting the ligand-binding domain, and A95 is located quite far from the pocket where the ligand interaction occurs but is in the vicinity of helices α4 and α5, which contribute to the ligand-binding domain.

Notably, 62% (29/47) of ETH^r isolates had mutations in *inhA* ($n = 6$) or the *inhA* promoter region ($n = 26$), which is in agreement with the results in other publications that reported that the proportions of strains carrying mutations in *inhA* or the *inhA* promoter ranged from 25 to 100% in ETH^r strains. The overall proportion is 68% (74/108) when the results from these publications are combined (4, 13, 17, 21, 24). These data confirm the significance of this mechanism in the structure or level of expression of the target InhA and in the development of resistance to ETH in clinical *M. tuberculosis* isolates. In our study, this mechanism is associated with mutations in EthA/EthR in a proportion of isolates equal to the proportion of isolates having mutations in *inhA* or the *inhA* promoter region without mutation in EthA/EthR.

In the two previous reports that tested *inhA*, the *inhA* promoter, and *ethA*, the proportions of strains with no mutation in these three genes were 21% (5/24) (4) and 7% (3/41) (21), which are close to the figures found in our study (9/47, 19%). We further investigated *mshA*, *ndh*, and *dfrA* in the nine ETH^r isolates lacking a mutation in the three main resistance genes. One double mutation (V171G-A187V) was detected in *mshA* in a single isolate; the other isolates had no mutation. The significance of the presence of this double mutation remains unclear because the *in vivo* selection of ETH^r/INH^r *mshA* mutants has been reported in only a single study (28) prior to the present study. For *ndh*, two reports have shown that *ndh* mutations in *M. smegmatis* and *M. bovis* BCG can mediate coresistance to ETH and INH (20, 30), but the impact of *ndh* mutations in *M. tuberculosis* is still unknown. The product of *dfrA*, DHFR, was proposed to be a new target for INH (1), but the consequences of *dfrA* mutations in clinical strains of *M. tuberculosis* are unknown. Taken together, the data suggest that the role played by *mshA*, *ndh*, and *dfrA* in ETH resistance in clinical strains of *M. tuberculosis* is unclear, and further studies are needed to investigate this role. ETH resistance likely stems from mechanisms that remain to be discovered, highlighting the complexity of the mechanisms underlying the resistance.

The critical concentrations and proportions chosen for DST were based on clinical failure; thus, strains with more than 10% resistant mutants were undoubtedly clinically resistant (8). Currently, strains with a proportion of resistant mutants of less than 10% are considered susceptible by WHO standards. Nevertheless, we extended our study to isolates with a high enough proportion (~1%) of ETH-resistant cells to be considered abnormal but not high enough (<10%) to be considered resistant according to WHO recommendations. Interestingly, 7 of the 16 ETH^{Sip} isolates displaying this pattern had *ethA* mutations known to be implicated in ETH^r; these corresponded to new mutations in 6 isolates, and 1 isolate had a mutation in *mshA*. Even though the proportion of isolates without a mutation was higher among ETH^{Sip} (8/16) than ETH^r (8/47) isolates ($P = 0.009$), the fact that isolates with >1% and <10% resistant mutants have mutations more often than fully susceptible isolates ($P = 0.004$) suggests that they should be regarded as isolates potentially resistant to ETH and might define a new clinically significant resistance category. To date, the clinical significance of these mutants remains unknown, but the existence of this class of isolates points to the potential danger of using a single cutoff for defining ETH resistance in phenotypic resistance assays.

Finally, among the 24 ETH^s isolates, only 1 harbored a mutation in the major genes known to be linked to ETH resistance. The consequence of the mutation (−47G → C) in the *inhA* promoter is unknown (10, 13, 24).

Because the rapid determination of drug resistance is an important prerequisite for the initiation of effective chemotherapy and preventing the acquisition of additional resistance traits, the fast prediction of resistance to bactericidal second-line drugs using DNA sequencing is of great interest, particularly in the context of MDR-TB. In the present study, mutations in *ethA*, *ethR*, *inhA*, or the *inhA* promoter region accounted for ETH resistance in 81% of the isolates. Consequently, finding such mutations by DNA sequencing or other molecular approaches can be expected to be a strong indicator of ETH resistance. However, the absence of such mutations should not be interpreted as indicating that a strain, particularly an MDR strain, is susceptible to ETH. Moreover, as long as other mechanisms of ETH resistance remain to be discovered, searching the strains for mutations in *dfrA*, *ndh*, and *mshA* will likely add limited value to the genetic detection of ETH resistance.

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