Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA

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Drug-resistant tuberculosis poses a significant problem for treatment. The mechanisms of resistance to the front-line drug isoniazid (INH) are complex and can be mediated by *katG*, *inhA* and other unknown genes. To identify the percentage of INH-resistant strains with no *katG* or *inhA* mutation, this study characterized a panel of 28 clinical isolates of *Mycobacterium tuberculosis* and five mutants derived from H37Rv resistant to INH. Seventeen of 33 resistant strains (51 %) had *katG* mutations with 12 of the 17 strains having the most common KatG Ser315Thr mutation. Three of the 17 strains with the KatG 315 mutation had an additional mutation in the *inhA* promoter and were resistant to a high level of INH. Seventeen of the 33 INH-resistant strains (51 %) had *inhA* mutations. The most common *inhA* promoter mutation was −15C→T and was present in 13 of the 17 *inhA* mutations. This promoter mutation occurred alone without *katG* mutations and was associated with a low level of INH and ethionamide resistance. However, other *inhA* mutations were associated with *katG* mutations. No mutations were found in the *ndh* gene. Three of 33 strains (9 %) had no mutations in *katG*, *inhA* or *ndh*, indicating that their resistance was due to a new mechanism of resistance. Detection of the KatG Ser315Thr mutation and the −15C→T *inhA* mutation accounted for 76 % (25/33) of the INH-resistant strains and should be useful for rapid detection of INH-resistant strains by molecular tests.

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INTRODUCTION

The increasing problem of drug-resistant, especially multi-drug-resistant, strains of *Mycobacterium tuberculosis* poses a significant threat to effective disease control in some parts of the world (Raviglione, 2003). Thus, there has been a great deal of interest in understanding the molecular mechanisms of drug resistance in this organism. Significant progress has been made in this area (Zhang *et al.*, 2005). Understanding the mechanisms of drug resistance in *M. tuberculosis* will facilitate the rapid molecular detection of drug-resistant strains and provide useful clinical guidance for appropriate treatment of the disease.

Isoniazid (INH) is an important first-line tuberculosis drug. *M. tuberculosis* is highly susceptible to INH, with an MIC of 0·03–0·06 μg ml^{-1} (Zhang, 2004). INH is a pro-drug that requires activation by the *M. tuberculosis* catalase-peroxidase enzyme (KatG) to its active form (Zhang *et al.*, 1992). Following activation, reactive radicals including isonicotinic acyl radical, isonicotinic acyl species (Rozwarski *et al.*, 1998; Broussy *et al.*, 2003) and reactive oxygen species (Shoeb *et al.*, 1985) can damage multiple targets in the cell (Zhang *et al.*, 2005). One of these targets is InhA, an NADH-dependent enoyl acyl carrier protein reductase involved in cell wall mycolic acid synthesis (Banerjee *et al.*, 1994).

Resistance to INH is mediated by at least two genes in *M. tuberculosis*, *katG* (Zhang *et al.*, 1992) and *inhA* (Banerjee *et al.*, 1994). Mutation of the *katG* gene, which leads to loss of or reduced catalase-peroxidase activity, is a major mechanism of INH resistance in *M. tuberculosis* (Heym *et al.*, 1995; Musser *et al.*, 1996; Zhang *et al.*, 2005). Although various mutations in the *katG* gene have been reported in INH-resistant isolates, the most common mutation is the KatG Ser315Thr mutation, which is present in approximately 50–90 % of all INH-resistant isolates and is associated with relatively high-level resistance to INH (Zhang *et al.*, 2005). Mutations in *inhA* or its promoter region can cause INH resistance, with promoter mutations being more frequent than mutations in the structural gene (Musser *et al.*, 1996). Mutations in InhA cause not only INH resistance, but also resistance to the structurally related second-line drug ethionamide (ETH) (Banerjee *et al.*, 1994).

Abbreviations: ETH, ethionamide; INH, isoniazid.

Although mutations in *kasA* encoding a β-keto-acyl-acyl carrier protein synthase involved in mycolic acid synthesis were initially found in INH-resistant strains (Mdluli *et al.*, 1998), subsequent studies found that *kasA* mutations were also detected in INH-susceptible strains (Lee *et al.*, 1999; Ramaswamy *et al.*, 2003). Mutations in *ndh*, encoding type II NADH dehydrogenase (Miesel *et al.*, 1998), which increases the NADH/NAD ratio and competes for the binding of activated INH (isonicotinic acyl radical) to the target InhA (Miesel *et al.*, 1998; Vilchèze *et al.*, 2005), have been found in some INH-resistant clinical isolates in only one study (Lee *et al.*, 2001). Mutations in the promoter region of *ahpC*, encoding alkyl hydroperoxide reductase, can compensate for loss of KatG in catalase-negative, INH-resistant strains (Sherman *et al.*, 1996; Wilson & Collins, 1996). However, overexpression of AphC does not appear to confer significant INH resistance and *ahpC* mutations may serve as a marker for INH resistance (Telenti *et al.*, 1997). Despite these advances, some INH-resistant strains, especially those with low- to intermediate-level resistance with positive catalase activity, do not have mutations in any of the above genes involved in INH resistance (Zhang *et al.*, 2005), suggesting a new mechanism(s) of INH resistance. In this study, we performed a detailed characterization of a panel of primarily INH-resistant *M. tuberculosis* strains in terms of their mechanism of INH resistance in order to shed light on the frequency of such strains.

METHODS

Mycobacterial growth. *M. tuberculosis* strains were grown in 7H9 liquid medium (Difco) supplemented with 0·05 % Tween 80 and 10 % bovine serum albumin/glucose/catalase enrichment (Difco) at 37 °C for approximately 2–3 weeks with occasional agitation.

Mycobacterial strains and drug-susceptibility testing. INH-resistant *M. tuberculosis* clinical isolates were obtained from New York State Department of Health, Albany, NY, USA. Strains R3, R8, R9, R10 and R11 were derived from *M. tuberculosis* H37Rv during *in vivo* treatment with INH in mice. The INH-resistant *M. tuberculosis* strains were identified by the BACTEC 460 radiometric method with an INH concentration of 0·1 μg ml^{-1} as the cut-off for resistance (Siddiqi, 1992). To determine the MICs of INH and ETH for the INH-resistant strains, the agar proportion method was performed in 7H11 plates containing varying concentrations of INH (0·2, 0·4, 1 and 5 μg ml^{-1}) or ETH (5 μg ml^{-1}).

Catalase activity assay. Catalase activity was assayed using a mixture of hydrogen peroxide (15 %) and Tween 80 (10 %) as described previously (Zhang *et al.*, 1993). *M. tuberculosis* H37Rv was included as a susceptible control strain in the drug-susceptibility testing and also as a positive control for the catalase assay.

Bacterial genomic DNA isolation, PCR and DNA sequencing. Genomic DNA was isolated as described previously (Zhang *et al.*, 1992). Oligonucleotide primers (Table 1) were designed from the *M. tuberculosis* H37Rv genome sequence (Cole *et al.*, 1998). The 2·2 kb *katG* gene (Rv1908c), a 1·5 kb region of the *mabA–inhA* gene (Rv1843–Rv1484) and the 1·4 kb *ndh* gene (Rv1854c) were amplified by PCR. The standard PCR mixture (50 μl) contained 1·5 U HotStarTaq DNA polymerase, 1× the recommended buffer supplemented with 1·5 mM $MgCl_2$ (Qiagen), 500 nM each forward and reverse primer, 200 μM each dATP, dGTP, dCTP and dTTP and 1 μl DNA template (~0·1 μg). PCR was performed using a Hybaid Omni-E PCR thermocycler with the following cycle conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 120 s, with a final extension at 72 °C for 10 min. PCR products were detected by 0·8 % agarose gel electrophoresis, followed by UV detection after ethidium bromide staining. To determine the *katG*, *inhA* and *mabA–inhA* promoter and *ndh* sequences, PCR products containing these genes were purified from the agarose gel after electrophoresis using a gel-purification kit (Qiagen) according to the manufacturer's instructions. PCR products were sequenced directly using an ABI 377 automatic DNA sequencer (Applied Biosystems) using appropriate primers for amplifying the INH resistance genes or internal sequencing primers (Table 1).

Table 1. Oligonucleotide primers used in PCR and DNA sequencing

INH resistance gene	Primer	Sequence (5′→3′)
katG	*katG*-F	TCCTGTTGGACGAGGCGGAG
	katG-R	CCGTCTCGTCATCCCCGTCT
	katG-S1	TGGGAGCCCGATGAGGTCTA
	katG-S2	AGATCCTGTACGGCTACGAG
	katG-S3	GGCGAAGCCGAGATTGCCAG
	katG-S4	ACAGCCACCGAGCACGAC
	katG-S5	GTCCCGTCATCTGCTGGCGA
	katG-S6	CCATGGGTCTTACCGAAAGT
mabA–inhA promoter	*inhA*-F	TCGTAGGGCGTCAATACACCGCA
	inhA-R	CGTCCAGCAGTCCTGTCATGTGCGT
ndh	*ndh*-F	ATTCACCGACGCCATCGACG
	ndh-R	ATGACACATGTCCTCAACTGG
	ndh-2F	TACTGGAATGGCTCACGCTC
	ndh-3F	CGCTGACCGAGCTTTGCGCC
	ndh-4F	AGGTCGATGTGGGTGACGTT

RESULTS AND DISCUSSION

Twenty-nine clinical isolates of *M. tuberculosis* resistant to 0·1 μg INH ml^{-1} were subjected to MIC determination by the 7H11 agar method as described in Methods (Table 2). Strain 2 was contaminated and was therefore discarded. The remaining 28 clinical isolates and five INH-resistant laboratory mutants derived from INH monotherapy of *M. tuberculosis* H37Rv infection in mice were analysed for their level of INH resistance, catalase activity and mutations in the genes associated with INH resistance. The level of INH resistance ranged from 0·2 to 5 μg ml^{-1}, with the majority of strains being resistant to 0·2–1 μg INH ml^{-1} (Table 2). Most of the strains were catalase-positive with low to intermediate levels of resistance. Four strains (strains 8, 27 and 28 and H37Rv mutant R9) had little or no catalase activity, with intermediate to high levels of resistance (1–5 μg ml^{-1}). Overall, the findings confirmed the previous observation that low- to intermediate-level resistant strains can be catalase-positive, whereas high-level resistant strains are often catalase-negative (Middlebrook, 1954; Zhang, 2004).

Sequence analysis revealed that 17 of the 33 strains (51 %) had *katG* mutations. Twelve of these 17 strains (strains 3, 5, 7, 9, 17, 18, 23, 26 and 29 and H37Rv mutants R8, R10 and R11) had the most common KatG Ser315Thr mutation, which retains catalase-peroxidase activity but causes reduced binding of INH to KatG (Wengenack *et al.*, 1998; Yu *et al.*, 2003). Strain 29, in addition to having the KatG Ser315Thr mutation, also had the KatG 463 polymorphism, which is not associated with INH resistance (Heym *et al.*, 1995). Strains with the KatG Ser315Thr mutation alone had MICs of ∼1 μg INH ml^{-1}. However, strains 3, 5 and 18, with mutations in the *inhA* promoter in addition to the KatG 315 mutation, were resistant to high levels of INH (5 μg ml^{-1}) (Table 2). Seventeen of the 33 INH-resistant strains (51 %) had *inhA* promoter or structural gene mutations (Table 2). The most common *inhA* promoter mutation was −15C→T (Ramaswamy *et al.*, 2003; Madison

Table 2. Characteristics of INH-resistant clinical isolates of *M. tuberculosis*

Strain	INH MIC (μg ml^{-1})	ETH*	Catalase activity	*inhA* promoter or structural gene mutation	*katG* mutations (nucleotide/amino acid changes)
H37Rv	<0·2	S	+++	−	−
1	0·2	R	+++	−15C→T	−
3	5	S	++	−8T→A	944G→C/315Ser→Thr
4	0·2	R	+++	−15C→T	−
5	1–5	S	++	−22G→C	944G→C/315Ser→Thr
6	0·2	R	+++	−15C→T	−
7	1	S	++	−	944G→C/315Ser→Thr
8	0·2	S	+/−	−	1226C→T/409Ala→Val
9	1	R	++	−	944G→C/315Ser→Thr
10	0·2	R	+++	−15C→T	−
11	0·2	R	+++	−15C→T	−
12	0·2	R	+++	−15C→T	−
13	0·2	R	+++	−15C→T	−
14	0·2	R	+++	−15C→T	−
15	0·4	R	+++	−15C→T	−
16	1–5	S	+++	−	−
17	1	S	+++	−	944G→C/315Ser→Thr
18	5	R	+++	−8T→C	944G→C/315Ser→Thr
19	0·2	R	+++	−15C→T	−
20	0·2	R	+++	−15C→T	−
21	0·2	R	+++	722C→T/241Thr→Met	1477A→C/493Asn→His
22	1	S	+++	−	−
23	1	S	+++	−	944G→C/315Ser→Thr
24	0·2	R	+++	−15C→T	−
25	0·2	R	+++	−15C→T	−
26	1	S	++	−	944G→A/315Ser→Asn, 1135G→A/379Ala→Thr
27	1	S	+	−	781G→C/261Glu→Gln
28	1	S	−	−	−
29	1	R	+++	−	944G→C/315Ser→Thr

*S, Sensitive; R, resistant (growth at 5 μg ETH ml^{-1}).

et al., 2004), which was present in 13 of the 17 *inhA* mutations. It is interesting to note that all 13 strains with the −15C→T mutation were resistant to a low level of INH (0·2 μg ml^{-1}) and did not have mutations in *katG* or *ndh*. In contrast, of the remaining four strains with *inhA* mutations (strains 3, 5, 18 and 21), three had *inhA* promoter mutations (−8T→A, −8T→C and −22G→C) and one had an *inhA* structural gene mutation of Thr241Met, which is a new mutation. These four strains also had mutations in *katG* in addition to *inhA* mutations (Table 2) and three of them (strains 3, 5 and 18) had the same KatG Ser315Thr mutation and were more resistant to INH (5 μg ml^{-1}). However, strain 21, which contained *inhA* Thr241Met and *katG* Asn493His, had a low MIC of 0·2 INH μg ml^{-1}. These findings suggest that: (i) −15C→T can be present by itself and is associated with a low level of INH resistance; (ii) *inhA* promoter mutations (e.g. −8T→A, −8T→C) can occur with *katG* mutations (KatG Ser315Thr) to confer a higher level of INH resistance; and (iii) mutation of the *inhA* structural gene is associated with a low level of resistance.

As *inhA* mutations also confer co-resistance to INH and ETH (Banerjee *et al.*, 1994), we examined the ETH susceptibility of the INH-resistant strains. Seventeen of the 33 strains were co-resistant to ETH, with all 13 strains with the *inhA* −15C→T mutation being resistant to both ETH and INH. Strains 18 and 21 with the −8T→C and Thr241Met *inhA* mutations, respectively, were also resistant to ETH. However, strains 3 and 5 with −8T→A and −22G→C *inhA* promoter mutations, respectively, were susceptible to ETH (Table 2). The *inhA* −15C→T mutation is one of the most commonly reported *inhA* mutations (Bakonyte *et al.*, 2003; Morlock *et al.*, 2003; Sajduda *et al.*, 2004) and presumably causes overexpression of InhA, the target of INH and ETH, thus resulting in co-resistance to both INH and ETH. Strains 9 and 29 were resistant to ETH but did not contain *inhA* or *ndh* mutations, and this could be due to mutations in *etaA*/*ethA* involved in ETH activation (Baulard *et al.*, 2000; DeBarber *et al.*, 2000). Further studies are needed to address the basis for mechanisms of ETH susceptibility in strains 3 and 5 with *inhA* mutations and also the ETH resistance in strains 9 and 29 without *inhA* mutations.

Mutation of *ndh*, which causes an increase in the NADH/NAD ratio, has been found to cause INH resistance in *Mycobacterium smegmatis* (Miesel *et al.*, 1998) and *Mycobacterium bovis* BCG (Vilchèze *et al.*, 2005), but not in *M. tuberculosis*, presumably because of the different role that Ndh plays in *M. bovis* compared with *M. tuberculosis* (Vilchèze *et al.*, 2005). So far, only one study has reported *ndh* mutations in some INH-resistant clinical isolates (Lee *et al.*, 2001), but unfortunately these strains were discarded and are not available for analysis of the stability of the *ndh* mutations. It is interesting to note that, in this study, of 33 INH-resistant strains, only one, strain 13, which had the −15C→T mutation in the *inhA* promoter, was initially found to harbour multiple mutations in the *ndh* gene. However, upon subculture in liquid medium without INH, strain 13 lost its *ndh* mutations. It is likely that strain 13 is composed of mixed bacterial populations of sensitive and resistant clones and that the clones harbouring mutations in *ndh* may be at a disadvantage and are therefore selected against during culture *in vitro*, although the clones with *ndh* mutations may survive *in vivo* and be detected initially when first isolated from clinical specimens.

Three (strains 16, 22 and 28) of the 33 strains (9 %) had no mutations in *katG*, *inhA* or *ndh*. Strains 16 and 22 were catalase-positive and their resistance could be due to a new mechanism of INH resistance. In contrast, strain 28 was catalase-negative and the mechanism of resistance in this strain is unknown but could result from mutations in the promoter or regulatory gene for *katG*. Further studies are needed to identify the new mechanisms of INH resistance in such strains.

It is worth noting that the five INH-resistant mutants derived from type strain H37Rv all had mutations in *katG*, with three having the characteristic KatG Ser315Thr mutation as a result of a G→C change at nt 944, one having a mutation of G→A at nt 544 leading to Gly182Arg and one having a mutation of C→T at nt 148 resulting in a stop codon, with negative catalase activity and a higher MIC (MIC >5 μg INH ml^{-1}).

It is of interest to note that, of the 33 INH-resistant strains, 12 had the KatG Ser315Thr mutation and 13 had the −15C→T *inhA* promoter mutation, accounting for 76 % (25/33) of the INH-resistant strains. Although the number of strains analysed was relatively small, the findings of this study are consistent with the results of other studies such as that of Baker *et al.* (2005), who found that 63 and 22 % of INH-resistant strains had the KatG Ser315Thr mutation and mutations in the *inhA* promoter, respectively. Molecular diagnostic tests based on detecting these two predominant mutations could be useful for the rapid detection of INH-resistant strains.

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