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 mutations, 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.

INTRODUCTION

The increasing problem of drug-resistant, especially multidrug-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).
Although mutations in \textit{kasA} encoding a \(\beta\)-keto-acyl-acyl carrier protein synthase involved in mycolic acid synthesis were initially found in INH-resistant strains (Mdluli \textit{et al.}, 1998), subsequent studies found that \textit{kasA} mutations were also detected in INH-susceptible strains (Lee \textit{et al.}, 1999; Ramaswamy \textit{et al.}, 2003). Mutations in \textit{ndh}, encoding type II NADH dehydrogenase (Miesel \textit{et al.}, 1998), which increases the NADH/NAD ratio and competes for the binding of activated INH (isonicotinic acid radical) to the target InhA (Miesel \textit{et al.}, 1998; Vilchèze \textit{et al.}, 2005), have been found in some INH-resistant clinical isolates in only one study (Lee \textit{et al.}, 2001). Mutations in the promoter region of \textit{ahpC}, encoding alkyl hydroperoxide reductase, can compensate for loss of KatG in catalase-negative, INH-resistant strains (Sherman \textit{et al.}, 1996; Wilson & Collins, 1996). However, overexpression of AphC does not appear to confer significant INH resistance and \textit{ahpC} mutations may serve as a marker for INH resistance (Telenti \textit{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 \textit{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 \textit{M. tuberculosis} strains in terms of their mechanism of INH resistance in order to shed light on the frequency of such strains.

\section*{METHODS}

\subsection*{Mycobacterial growth.} \textit{M. tuberculosis} strains were grown in \textit{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.

\subsection*{Mycobacterial strains and drug-susceptibility testing.} INH-resistant \textit{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 \textit{M. tuberculosis} H37Rv during \textit{in vivo} treatment with INH in mice. The INH-resistant \textit{M. tuberculosis} strains were identified by the BACTEC 460 radiometric method with an INH concentration of 0.1 \(\mu\)g 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 \textit{7H11} plates containing varying concentrations of INH (0.2, 0.4, 1 and 5 \(\mu\)g ml\(^{-1}\)) or ETH (5 \(\mu\)g ml\(^{-1}\)).

\subsection*{Catalase activity assay.} Catalase activity was assayed using a mixture of hydrogen peroxide (15\%) and Tween 80 (10\%) as described previously (Zhang \textit{et al.}, 1993). \textit{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.

\subsection*{Bacterial genomic DNA isolation, PCR and DNA sequencing.} Genomic DNA was isolated as described previously (Zhang \textit{et al.}, 1992). Oligonucleotide primers (Table 1) were designed from the \textit{M. tuberculosis} H37Rv genome sequence (Cole \textit{et al.}, 1998). The 2.2 kb \textit{katG} gene (Rv1908c), a 1.5 kb region of the \textit{mabA}–\textit{inhA} gene (Rv1848c–Rv1484) and the 1.4 kb \textit{ndh} gene (Rv1854c) were amplified by PCR. The standard PCR mixture (50 \(\mu\)l) contained 1.5 \(\mu\)l HotStarTaq DNA polymerase, 1 \(\times\) the recommended buffer supplemented with 1.5 mM MgCl\(_2\) (Qiagen), 500 nM each forward and reverse primer, 200 \(\mu\)M each dATP, dGTP, dCTP and dTTP and 1 \(\mu\)l DNA template (\(\sim\)0.1 \(\mu\)g). PCR was performed using a Hybaid Omnii-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 10 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 \textit{katG}, \textit{inhA} and \textit{mabA}–\textit{inhA} promoter and \textit{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).

\begin{table}[h]
\centering
\caption{Oligonucleotide primers used in PCR and DNA sequencing}
\begin{tabular}{|l|l|l|}
\hline
\textbf{INH resistance gene} & \textbf{Primer} & \textbf{Sequence (5’\textit{→}3’)} \\
\hline
\textit{katG} & \textit{katG-F} & TCCGTGTGGAGCGGAGAGGAG \\
 & \textit{katG-R} & CCGTCTGCTGCTACCCGCTCT \\
 & \textit{katG-S1} & TGGGAGCGCCGAGGCTCTA \\
 & \textit{katG-S2} & AGATCCTGTAAGGTGAGGAG \\
 & \textit{katG-S3} & GCGGAGCTGAGTGTGCGAG \\
 & \textit{katG-S4} & ACAGCCACCAGGACGAC \\
 & \textit{katG-S5} & GTCCGCTGCTGCTGAGG \\
 & \textit{katG-S6} & CCATGTCGCTCCGAAAGT \\
 & \textit{mabA–inhA promoter} & \textit{inhA-F} & TCAGTGAGGCGTCCTATACCCGCA \\
 & \textit{inhA-R} & CGTCCGACGCTGTCATGTCGCT \\
 & \textit{ndh} & \textit{ndh-F} & ATTCACGACGGCATCCGACG \\
 & \textit{ndh-R} & ATGACAGAGTCGTCCTCAACTTG \\
 & \textit{ndh-2F} & TACTGGAATGCTCAGGTC \\
 & \textit{ndh-3F} & CGTTCGACGGGCTTTGCCG \\
 & \textit{ndh-4F} & AGGTCGATGCGGTCAGGTT \\
\hline
\end{tabular}
\end{table}
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*

<table>
<thead>
<tr>
<th>Strain</th>
<th>INH MIC (μg ml⁻¹)</th>
<th>ETH*</th>
<th>Catalase activity</th>
<th><em>inhA</em> promoter or structural gene mutation</th>
<th><em>katG</em> mutations (nucleotide/amino acid changes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>&lt;0.2</td>
<td>S</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>S</td>
<td>+</td>
<td>−8T→A</td>
<td>944G→C/315Ser→Thr</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>R</td>
<td>+</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>1–5</td>
<td>S</td>
<td>+</td>
<td>−22G→C</td>
<td>944G→C/315Ser→Thr</td>
</tr>
<tr>
<td>6</td>
<td>0.2</td>
<td>R</td>
<td>+</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>S</td>
<td>+</td>
<td>−</td>
<td>944G→C/315Ser→Thr</td>
</tr>
<tr>
<td>8</td>
<td>0.2</td>
<td>S</td>
<td>+/−</td>
<td>−</td>
<td>1226C→T/409Ala→Val</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>R</td>
<td>+</td>
<td>−</td>
<td>944G→C/315Ser→Thr</td>
</tr>
<tr>
<td>10</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>11</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>12</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>13</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>16</td>
<td>1–5</td>
<td>S</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>S</td>
<td>+++</td>
<td>−</td>
<td>944G→C/315Ser→Thr</td>
</tr>
<tr>
<td>18</td>
<td>5</td>
<td>R</td>
<td>+++</td>
<td>−8T→C</td>
<td>944G→C/315Ser→Thr</td>
</tr>
<tr>
<td>19</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>21</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>722C→T/241Thr→Met</td>
<td>1477A→C/493Asn→His</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>S</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>23</td>
<td>1</td>
<td>S</td>
<td>+++</td>
<td>−</td>
<td>944G→C/315Ser→Thr</td>
</tr>
<tr>
<td>24</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>25</td>
<td>0.2</td>
<td>R</td>
<td>+++</td>
<td>−15C→T</td>
<td>−</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>S</td>
<td>+</td>
<td>−</td>
<td>944G→A/315Ser→Asn, 1135G→A/379Ala→Thr</td>
</tr>
<tr>
<td>27</td>
<td>1</td>
<td>S</td>
<td>+</td>
<td>−</td>
<td>781G→C/261Glu→Gln</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>S</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>29</td>
<td>1</td>
<td>R</td>
<td>+++</td>
<td>−</td>
<td>944G→C/315Ser→Thr</td>
</tr>
</tbody>
</table>

*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 (Vilčhež et al., 2005), but not in M. tuberculosis, presumably because of the different role that NdH plays in M. bovis compared with M. tuberculosis (Vilčhež 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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REFERENCES


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