INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Ellard & Gammon, 1976) and is effectively treated with isoniazid (INH) (Torres et al., 2000). Thus, resistance to INH is a cause for concern. A recent WHO/IUATLD (International Union Against Tuberculosis and Lung Disease) survey of drug resistance to TB showed that 14.4% of (International Union Against Tuberculosis and Lung Disease) INH-resistant cases, and 5.9% of the new cases were resistant to INH (WHO, 2004). It is thought that once INH resistance occurs, resistance to other drugs frequently follows, leading to multidrug-resistant TB (MDR TB), for which treatment is expensive and not undertaken by many poorer nations (Grange & Zumla, 2002). The median percentage of MDR TB is 1.1% in the majority of the previously treated cases and 9.9% of the new cases were resistant to INH (WHO, 2004). It is thought that once INH resistance occurs, resistance to other drugs frequently follows, leading to multidrug-resistant TB (MDR TB), for which treatment is expensive and not undertaken by many poorer nations. The median percentage of MDR TB is 1.1% in the majority of the previously treated cases and 9.9% of the new cases were resistant to INH (WHO, 2004). It is thought that once INH resistance occurs, resistance to other drugs frequently follows, leading to multidrug-resistant TB (MDR TB), for which treatment is expensive and not undertaken by many poorer nations.

A gene (nat) encoding arylamine N-acetyltransferase (NAT) has been found in *Mycobacterium tuberculosis*. The gene is expressed and the enzyme is active in growing *M. tuberculosis* cells. *N*-Acetyltransferase acetylates and inactivates isoniazid (INH), which is a front-line drug used in tuberculosis (TB) therapy. In this study, it was shown that a previously reported G619A single nucleotide polymorphism (SNP) was conserved in two *M. tuberculosis* strain families found in the Western Cape Province of South Africa (strain families 3 and 28). Further sequence analysis of isolates in strain family 3 identified a new T529C SNP in NAT resulting in a histidine instead of a tyrosine at position 177. This SNP was found only in isolates from strain family 3, and this mutation affects the highly conserved tyrosine residue close to the active site. Using real-time PCR, the expression of *M. tuberculosis* nat (*tnat*) was determined over a 28 day growth cycle of the *M. tuberculosis* reference strain (H37Rv). The expression of *tnat* occurs early in growth and reaches maximum levels at mid-exponential phase. The exposure of INH susceptible isolates to low levels of INH resulted in an increase of *tnat* expression (reference strain H37Rv, which is wild-type for *tnat*, and isolate 1430, containing both SNPs). An INH-resistant isolate (816) exposed to INH showed no change in *tnat* expression. The increased expression in the susceptible isolates suggests that INH affects *tnat* expression. *tnat* may contribute to INH susceptibility, but in combination with other factors.

The arylamine *N*-acetyltransferases (NATs) catalyse the transfer of the acetyl group from acetyl coenzyme A to the terminal nitrogen of an aromatic amine, a heterocyclic amine or a hydrazine compound (Weber & Hein, 1985). The active-site catalytic triad consisting of cysteine, histidine and aspartate has been shown to be highly conserved in prokaryotic and eukaryotic NAT homologues, together with three highly conserved regions which are found proximal to the active-site triad (Sim et al., 2000; Sandy et al., 2005; Payton et al., 2001b). Activation of INH in *M. tuberculosis* by catalase-peroxidase (encoded by katG) involves oxidation of the hydrazine moiety, which cannot occur when INH is *N*-acetylated (Upton et al., 2001). Human NAT2 inactivates INH by acetylation, and different polymorphisms in this gene have functional effects on the enzyme (conferring fast-, intermediate- or slow-acetylator status to individuals) (Par-
A gene encoding NAT has been found in *M. tuberculosis* (Payton et al., 1999; Upton et al., 2001).

Recombinant nat from *M. tuberculosis* (tbnat) has been shown to N-acetylate INH in vitro, and when the *M. tuberculosis* nat gene is overexpressed in *Mycobacterium smegmatis*, the resistance to INH of the transformed organism increases threefold (Payton et al., 1999). These results suggest that tbnat may be involved in INH susceptibility. The gene is expressed in *M. tuberculosis* and the enzyme is active (Upton et al., 2001). There is evidence to suggest that tbnat encodes a protein that is important for growth and development, as well as normal mycolic acid synthesis and hence other derivatives of cell wall components (Bhakta et al., 2004). This implies that tbnat may be used as a novel target for drug therapy (Payton et al., 1999; Bhakta et al., 2004). tbnat, like its human homologue, has been shown to be polymorphic, and a G619A single nucleotide polymorphism (SNP) has been identified in the ORF of this gene in some *M. tuberculosis* isolates (Upton et al., 2001). The polymorphism, which is within the coding region, adversely affects the activity of the enzyme, such that the recombinant enzyme with an arginine residue at position 207 has a fourfold lower apparent affinity and the rate of activity is greatly reduced.

In order to further investigate whether tbnat is involved in INH resistance in clinical isolates of *M. tuberculosis*, we searched extensively for SNPs and studied the expression of tbnat during the growth cycle of *M. tuberculosis*, as well as investigating whether INH has an effect on the expression of tbnat in growing cultures of *M. tuberculosis*.

### METHODS

**Mycobacterium tuberculosis isolates.** A collection of genomic DNA samples and a corresponding database, with clinical and molecular information on *M. tuberculosis* clinical isolates, was available for this study (Warren et al., 2000). The clinical isolates were obtained from TB patients residing in the Western Cape Province of South Africa. The isolates have been grouped into at least 37 strain families and 230 unique isolates, where a family of strains is defined as strains which are >65% related in terms of RFLP IS6110 DNA banding patterns, and there are also isolates with unique IS6110 patterns which do not fall into any of the families (Warren et al., 2000). The INH-resistance status of many of the samples in the database is known; however, the precise INH MIC and mutation status of all of these isolates is not known.

**MICs of selected strains.** Clinical isolates (Table 1) were selected from the available database according to their INH-resistance status and tbnat G619A variant status. Four INH-resistant isolates, four isolates from strain family 3, six isolates from strain family 28 (including one of the INH-resistant strains), one INH-susceptible isolate from strain family 11 and the reference strain H37Rv (Table 1) were selected for further MIC analysis (Warren et al., 2000). Isolates from strain family 3 and strain family 28 contain the tbnat G619A SNP (this study identified that strain family 3 had an additional T529C SNP; see subsequent sections). These isolates were grown on BACTEC medium and the MICs of INH were determined using concentrations of 0.005, 0.0125, 0.025, 0.05, 0.1, 1, 5 and 10 µg INH ml⁻¹, as described in the BACTEC manual.

### Table 1. Polymorphisms, mutations and MIC of INH in different isolates of *Mycobacterium tuberculosis*

A collection of DNA samples and a corresponding database with clinical and molecular information from *M. tuberculosis* clinical isolates, obtained from TB patients residing in the Western Cape Province of South Africa, was used for this study (Warren et al., 2000). From this collection, isolates (already classified into specific strain families) were selected according to their SNP and mutational status and whether they were resistant to INH or not. Isolates with an INH MIC higher than 0.1 µg ml⁻¹ were regarded as resistant. INH-resistant isolates were sequenced in regions of genes in which INH-associated mutations have been previously described (*katG, inhA, kasA, aphC* and *ndh; Ramaswamy et al., 2003*). Identified mutations are shown in the Mutational status column. +, Presence; -, absence.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Strain family</th>
<th>Mutational status</th>
<th>nat G619A SNP</th>
<th>nat T529C SNP</th>
<th>MIC (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>Not classified</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>131</td>
<td>2</td>
<td>+katG (315AGC-AAC)</td>
<td>–</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>929</td>
<td>3</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0.025</td>
</tr>
<tr>
<td>1394</td>
<td>3</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0.05</td>
</tr>
<tr>
<td>1398</td>
<td>3</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0.05</td>
</tr>
<tr>
<td>1430</td>
<td>3</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0.025</td>
</tr>
<tr>
<td>208</td>
<td>11</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.025</td>
</tr>
<tr>
<td>766</td>
<td>18</td>
<td>+katG (315AGC-ACC)</td>
<td>–</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>404</td>
<td>28</td>
<td>+katG (315AGC-ACC)</td>
<td>+</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>83</td>
<td>28</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>0.05</td>
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<tr>
<td>526</td>
<td>28</td>
<td>–</td>
<td>+</td>
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<td>704</td>
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<td>–</td>
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<td>–</td>
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<td>+</td>
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<td>1595</td>
<td>28</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>0.05</td>
</tr>
<tr>
<td>816</td>
<td>133</td>
<td>+aphC promoter (–9 G-A)</td>
<td>–</td>
<td>–</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

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On: Mon, 11 Feb 2019 20:03:22 Journal of Medical Microbiology 54
Sequence analysis of the M. tuberculosis isolates. In the INH-resistant isolates, each gene (katG, inhaA, kasA, ahpC and ndh) known to have mutations associated with INH resistance was sequenced (Ramaswamy & Musser, 1998; van Rie et al., 2001; Lee et al., 2001). The primers used for the amplification and sequencing of these genes are shown in Table 2. The ORF and immediate upstream and downstream regions of the tbnat gene of five isolates were amplified and sequenced (for primers, see Table 2). The five isolates consisted of two INH-susceptible isolates (H37Rv and isolate 208; Table 1), two INH-resistant isolates (isolates 131 and 816; Table 1) and one INH-susceptible isolate from strain family 3 (isolate 1430). The isolates found in strain family 3 have the G619A SNP, but unlike isolates in strain family 28, the tbnat gene in this strain family had not yet been screened for mutations by sequence analysis. The INH-resistant isolates were selected because their MICs for INH were high and they did not belong to strain family 3 or strain family 28.

DNA sequencing was carried out using an ABI 3100 instrument, using Bigdye chemistry (Applied Biosystems). Sequence alignments to the corresponding gene sequence of M. tuberculosis H37Rv [Rv3566c (Cole et al., 1998)] were done using DNAMAN version 4.0 (Lynnon BioSoft, copyright 1994–1997).

Analysis of the G619A NAT and T529C SNPs. PCR–RFLP analysis, using the restriction enzymes BsmAI and BglI, was used to screen M. tuberculosis DNA for the G619A (Upton et al., 2001) and T529C SNPs, respectively. A total of 468 representative clinical isolates obtained from the M. tuberculosis collection were used to screen for the G619A SNP, and 250 clinical isolates were used to screen for the T529C SNP (identified in this study). These isolates represented all 37 strain families as well as 10 unique isolates (isolates that could not be classified into a strain family). The primers used and the PCR conditions to detect both

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG first fragment</td>
<td>RTB S1</td>
<td>CAGAAACACCACCCGAGGCC</td>
<td>59</td>
<td>945</td>
</tr>
<tr>
<td>katG second fragment</td>
<td>RTB S9</td>
<td>GCTGGTGATGCTGCTCTTAC</td>
<td>66</td>
<td>804</td>
</tr>
<tr>
<td>inhaA promoter</td>
<td>inha P5</td>
<td>CGAGAGCCGAGGCTCGTG</td>
<td>60</td>
<td>246</td>
</tr>
<tr>
<td>inhaA</td>
<td>inha P3</td>
<td>CTCGGTAACAGAGCTGGA</td>
<td>64</td>
<td>169</td>
</tr>
<tr>
<td>ahpC promoter</td>
<td>ahpC S1</td>
<td>GCTGATGTGACCAGACCTG</td>
<td>60</td>
<td>701</td>
</tr>
<tr>
<td>kasA gene</td>
<td>kasA S1</td>
<td>ATTGAGTCCGAGAACCCCGA</td>
<td>56</td>
<td>1389</td>
</tr>
<tr>
<td>ndh gene</td>
<td>ndh3s</td>
<td>GACAGTGCCCGAGCTGCCC</td>
<td>60</td>
<td>372</td>
</tr>
<tr>
<td>Upstream nat gene</td>
<td>TBNat3a</td>
<td>CCTGCGAAACATCAGGTGTT</td>
<td>60</td>
<td>820</td>
</tr>
<tr>
<td>ORF nat gene</td>
<td>nat5</td>
<td>GACAGGTCATAATGGCAAAC</td>
<td>60</td>
<td>928</td>
</tr>
<tr>
<td>Downstream nat gene</td>
<td>TBNat1</td>
<td>TGGAAATCGGAGGTTGGGAG</td>
<td>60</td>
<td>1122</td>
</tr>
</tbody>
</table>

Effects of INH on tbnat expression. Three isolates (H37Rv, isolate 1430 and isolate 816; Table 1) were selected, as they represented a reference strain, an isolate with both SNPs and an isolate resistant to INH with an INH MIC greater than 10 µg ml⁻¹. These isolates were cultured in 7H9 medium, with stirring, to an OD₆₀₀ of 0.2–0.4. The cultures were separated into two aliquots and INH to a concentration of 0.01 µg ml⁻¹ (ten times lower than the critical concentration used for susceptibility testing in BACTEC) was added to one aliquot of each of the cultures. The remaining cultures were used as corresponding controls.
controls. RNA was extracted (Upton et al., 2001) at various time points over a 20 h period. The relative amounts of cDNA for tbnat and 16S rRNA were quantified as described above with an ABI Prism 7700 Sequence Detection System. The tbnat expression levels were calculated relative to 16S rRNA (Vandecasteele et al., 2001). It has previously been shown that INH does not appear to alter the expression of 16S rRNA (Hellyer et al., 1999; Alland et al., 1998), making 16S rRNA a good endogenous control for these experiments.

RESULTS

The previously identified G619A SNP in tbnat is restricted to two strain families

The G619A SNP previously identified in tbnat introduces an additional restriction site for BsmAI, which enabled numerous isolates to be tested for this SNP using PCR–RFLP. A total of 468 isolates representing 37 strain families and 10 unique isolates from the database in the Western Cape of South Africa were tested for this SNP. This SNP was found to occur in all isolates of strain family 28 (n = 187), strain family 3 (n = 6) and one unique isolate (isolate number 1936). No isolates from other strain families or unique isolates of M. tuberculosis had this SNP. The G619A SNP is, therefore, restricted to two strain families from the Western Cape TB communities. This mutation causes a Gly residue at position 207 to change to an Arg and results in a fourfold reduction in enzyme activity, and is likely to cause a change in the three-dimensional structure of the enzyme resulting in a change in the juxtaposition of amino acid residues important for enzyme activity (Upton et al., 2001) (Fig. 1).

Sequence analysis of M. tuberculosis isolates and identification of a new SNP, T529C

Mutations in the following genes, katG, inhA, kasA, ahpC and ndh, have been associated with INH resistance. The information for some of these mutations is included in the database of clinical isolates used in this study. An INH-resistance-associated mutation was identified in all four INH-resistant isolates that were sequenced. Three of these mutations occurred in the katG gene at codon 315 (Table 1), and one occurred in the aphC promoter region (Table 1).

To search for other nucleotide changes in the immediate upstream, downstream or ORF regions of the tbnat gene, five clinical isolates were selected for extensive sequence analysis of these regions (Table 2). No polymorphisms were identified within 791 bp upstream of the 5’ end or within 1122 bp downstream of the 3’ end of the tbnat gene. However, an additional T529C SNP was identified in the tbnat ORF of isolate 1430 of strain family 3. This SNP resulted in a Y177H amino acid change and could be detected using the restriction enzyme BglI. This restriction enzyme discriminates for the T529C SNP when the mutation is present: two DNA fragments of 546 and 382 bp are produced; otherwise, a single fragment of 926 bp is present.

A total of 250 isolates, representing 37 strain families and 10 unique isolates from the database in the Western Cape of South Africa were tested for this SNP using PCR–RFLP. A total of 468 isolates representing 37 strain families and 10 unique isolates from the database in the Western Cape of South Africa were tested for this SNP. This SNP was found to occur in all isolates of strain family 28 (n = 187), strain family 3 (n = 6) and one unique isolate (isolate number 1936). No isolates from other strain families or unique isolates of M. tuberculosis had this SNP. The G619A SNP is, therefore, restricted to two strain families from the Western Cape TB communities. This mutation causes a Gly residue at position 207 to change to an Arg and results in a fourfold reduction in enzyme activity, and is likely to cause a change in the three-dimensional structure of the enzyme resulting in a change in the juxtaposition of amino acid residues important for enzyme activity (Upton et al., 2001) (Fig. 1).
South Africa, were tested for the T529C SNP using RFLP analysis (enzyme BsgI). This SNP was found to occur in all isolates of strain family 3 ($n = 6$). No isolates from the other families or unique strains of *M. tuberculosis* had this SNP. The T529C SNP appears, therefore, to be restricted to strain family 3, and it confers an amino acid change from Tyr at position 177 to His.

**INH MICs in selected *M. tuberculosis* isolates**

MIC analysis (Table 1) shows that isolate 816 is highly resistant to INH at a concentration greater than 10 $\mu$g ml$^{-1}$. Although a mutation in the *aphC* promoter region has been identified in this isolate, it is possible that additional mutations at other loci may be present, resulting in this high MIC for INH. Analysis of isolates with the G619A and T529C SNPs compared to the reference strain H37Rv (no mutation in *nat* present) showed an increase in the MIC from less than 0.005 for H37Rv to 0.05 $\mu$g ml$^{-1}$ in isolates from families 3 and 28 (i.e. isolate 1430 with both the G619A and the T529C SNPs). This change in the MIC is not significant, and suggests that the G619A and T529C SNPs alone contribute only marginally to defining INH susceptibility or resistance status.

The Tyr$^{177}$!His$^{177}$ mutation was modelled, and all rotomers of the histidine residue were analysed for clashes or interactions. No rotomers of the histidine residue were able to reproduce the hydrogen bonding with Asp$^{127}$ that is observed with the isoform of the wild-type of the NAT enzyme with a tyrosine at position 177 (Fig. 1C, D).

**Expression of tbnat**

During the growth cycle of the *M. tuberculosis* strain H37Rv, *tbnat* mRNA levels are detected early, peaking at mid-exponential phase, after which the *tbnat* levels decline. By stationary phase, *tbnat* is being expressed at much lower levels.
levels than at mid-exponential phase (Fig. 2). Using OD_{600} and protein concentration as an estimate of cell number produced the same tbnat expression pattern.

**INH and tbnat expression**

The presence of non-bactericidal levels of INH in growing *M. tuberculosis* cultures results in an increase in tbnat expression; however, the extent of this increase differs between isolates (Fig. 3). Results were discarded after 6 h of INH exposure as the results became ambiguous due to cell death, especially with strain H37Rv. Strain H37Rv, the isolate most sensitive to INH, shows a rapid and extensive increase in tbnat expression up to 2 h, whereas isolate 1430, a strain which contains both the tbnat T529C and G619A SNPs, shows a more gradual increase in tbnat expression up to 2 h. Isolate 816, an isolate that is highly resistant to INH (MIC >10 μg ml⁻¹), does not show any increase in tbnat expression after exposure to INH. After 2 h INH exposure, strain H37Rv showed a 30-fold increase in tbnat expression, whereas isolate 1430 showed a sixfold increase. (Fig. 3)

**DISCUSSION**

A gene (tbnat) encoding arylamine N-acetyltransferase has been discovered in *M. tuberculosis* (Payton et al., 1999; Upton et al., 2001). *M. smegmatis* transformants induced to express the tbnat gene in culture demonstrate a threefold higher resistance to INH (Payton et al., 1999), suggesting a role in INH resistance. In a more recent study, it has been shown that *Mycobacterium bovis* BCG nat may play a critical role in the synthesis of mycolic acid and hence of derivatives of cell wall components, which represents a novel role for nat in mycobacteria. These findings suggest that nat in mycobacteria could be a target for drug therapy (Bhakta et al., 2004).

A SNP has been identified in a randomly selected number of clinical isolates at nucleotide 619 of this gene (Upton et al., 2001). In the work reported here, the SNP was further investigated to determine how widespread it is in clinical isolates and to assess whether clinical isolates with this polymorphism have a higher level of resistance to INH than those isolates without the SNP. Upon sequencing of the nat gene of selected isolates and of its upstream and downstream regions, a new SNP was identified in the ORF of an isolate from family 3. The previously identified G619A SNP was restricted to isolates in strain families 3 and 28, as well as to one unique isolate, whereas the newly identified T529C SNP was restricted to strain family 3. The G619A SNP constitutes 20 % and the T529C SNP constitutes 2-4 % of the clinical isolates tested in our environment. These nonsynonymous
SNPs (Gutacker et al., 2002) may be used instead of IS6110 profiling to identify these specific groups of clinical isolates, i.e. they may be used as molecular markers for epidemiological studies for certain strain families.

Drug-resistant isolates are found in many strain families that do not have these SNPs, and no significant differences in INH resistance were obtained from the MICs of isolates with or without the SNPs. The G207R mutation reduces the activity of the NAT enzyme towards INH, and the mutation which causes Y177R is likely to reduce the activity of NAT. Therefore, these mutations alone would be expected to increase the sensitivity of the strains to INH. It is clear that these mutations alone do not control INH sensitivity, and it is likely that a combination of mutations results in the level of INH sensitivity observed in different strains.

Targeted disruption of nat in M. smegmatis and gene deletion in M. Bovis BCG result in a delayed entry of the cells into exponential-phase growth, extending the length of the lag phase. This suggests that the presence of nat is required during early growth (Payton et al., 2001a; Bhakta et al., 2004).

The expression of tbnat in M. tuberculosis strain H37Rv was monitored over 28 days. The results were obtained from three separate growing cultures, which made it impossible to extract exact growth stages at each time point. This is evident in the variability obtained for both the relative tbnat expression and the OD_{600} (Fig. 2). The results from this study show that the gene is expressed during the initial stages of cell growth, with the maximum expression levels being obtained at mid-exponential phase, which occurs around day 7. As H37Rv reaches stationary phase, the expression of tbnat drops. Expression early in the growth cycle supports the suggestion that tbnat is important in growth.

When M. tuberculosis cultures of three different isolates were exposed to INH, each showed an increase in tbnat expression, although the relative increase of tbnat mRNA varied amongst the three isolates. The reference strain, which is highly sensitive to INH (MIC < 0.01 µg ml^{-1}), showed a rapid increase in tbnat expression up to 2 h (30-fold increase after 2 h) of INH exposure, while the isolate containing the SNPs had an increased MIC (0.025–0.05 µg ml^{-1}) and showed a more gradual increase in tbnat expression (sixfold increase after 2 h) and the isolate with high-level INH resistance (MIC >10 µg ml^{-1}) showed no increase in tbnat expression. These results suggest that INH affects the expression of tbnat in susceptible isolates. From these results it cannot be determined whether INH interacts directly with the promoter of tbnat or whether changes in the cell environment cause the increase in tbnat expression. In the resistant isolate studied (with aphC promoter mutation, −9 G-A), INH had little effect on tbnat expression. This difference between the sensitive and resistant isolates can only be explained once the mechanism by which INH interacts with the nat gene is understood.

It has been reported that the tbnat gene is part of an operon involving four other genes (Payton et al., 2001a). A fifth gene has recently been added to this operon (NCBI, 2004). The promoter region would be expected to lie upstream of this operon. It may also be the case that more than one factor is involved in the control of tbnat expression. For example, more than one promoter could be involved in tbnat expression, as discovered for the katG gene and the rRNA operon (Gonzalez-y-Merchand et al., 1998; Master et al., 2001). The promoters may function independently and respond to varied environmental inputs and physiological demands (Master et al., 2001; Gonzalez-y-Merchand et al., 1998), such as cell-wall-synthesis requirements. It could also be the case that one of the other genes in the operon may need to be expressed before the expression of tbnat can occur. The expression of the other genes in the proposed operon therefore needs to be investigated further. Bioinformatics analysis has recently placed the promoter downstream of the first gene of the operon in which nat is predicted to be transcribed (Anderton et al., 2004).

There is evidence that INH alters the expression of a range of genes in M. tuberculosis strain H37Rv (Wilson et al., 1999), and these include genes which are involved in cell wall synthesis, such as those of the antigen 85 complex. As for tbnat, the genes (especially fabC) encoding the antigen 85 complex have been shown to be induced in strain H37Rv, but are not induced in INH-resistant M. tuberculosis isolates (Garbe et al., 1996; Wilson et al., 1999). As determined in microarray studies, in which genes upregulated by INH were investigated, the tbnat gene does not appear to be induced in the presence of INH (Wilson et al., 1999). The isolates and methods presented here give additional information to aid the identification of candidate genes that may not have been detectable in the microarray study due to relatively low levels of mRNA (Vainrub & Montgomery Petitt, 2003). The combination of microarray analysis together with a candidate gene approach provides complementary information. The fabC gene, as well as a number of other genes that show increased expression levels in cells exposed to INH (Wilson et al., 1999), has been sequenced, and no INH-resistance-associated mutations have been identified in any of the following genes: fabC, fabD, accD6, efpA and ndh (Ramaswamy et al., 2003).

This study shows that the tbnat gene is conserved, and that polymorphisms in this gene are found in only a minority of clinical isolates. The SNPs studied here imply that changes in the tbnat gene are strain-family specific and may be used in epidemiological studies. It has also been shown that tbnat is expressed early in the growth cycle of M. tuberculosis, and that the exposure of growing M. tuberculosis cultures to INH results in an increase in tbnat expression. It is still not clear whether this gene plays a role in INH resistance; however, these results do add to previous findings (Payton et al., 1999, 2001a; Upton et al., 2001) suggesting that tbnat and INH do interact with each other. Before the significance of this interaction can be determined, the mechanism by which INH interacts with the nat gene and its product needs to be fully understood.
ACKNOWLEDGEMENTS

We thank the Department of Science and Technology/National Research Foundation (DST/NRF) for funding assistance to the Centre of Excellence for Biomedical TB Research and The Wellcome Trust for funding work in E.S.’s laboratory.

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