Evaluation of gidB alterations responsible for streptomycin resistance in Mycobacterium tuberculosis

Jitender S. Verma1*, Yash Gupta2, Deepthi Nair3, Nikhat Manzoor4, Rajinder S. Rautela5, Arvind Rai5 and Vishwa M. Katoch6

1Department of Orthopaedics, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi 110029, India; 2National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Tajganj, Agra 282001, India; 3Department of Microbiology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi 110029, India; 4Department of Bio-Sciences, Jamia Millia Islamia, New Delhi 110025, India; 5National Centre for Disease Control, Sham Nath Marg, New Delhi 110054, India; 6Director General, Indian Council of Medical Research and Secretary, Department of Health Research, Ministry of Health and Family Welfare, India

*Corresponding author. E-mail: jitu_jitender1@rediffmail.com

Received 13 March 2014; returned 9 June 2014; revised 17 June 2014; accepted 20 June 2014

Objectives: To evaluate gidB alterations for possible impact on the cumulative mechanism underlying the acquisition of high-level streptomycin resistance in Mycobacterium tuberculosis.

Methods: Fifty-two isolates with high streptomycin resistance and 23 isolates with low streptomycin resistance were sequenced for mutational analysis in the rpsL, rrs and gidB region. As the gidB protein has a complex substrate and no activity assay has yet been formulated, mutants of interest were subjected to in silico modelling and were structurally mapped together with active-site amino acid residues for assessment of the relevance to activity of the mutations found.

Results: Eight novel sense mutations and four novel mis-sense mutations in gidB were identified. Findings showed that active-site morphology is not only greatly affected by mutants lying in close proximity to the active-site pocket, but also by other mutations altering secondary-structure motifs and having an overall effect on protein structure.

Conclusions: We conclude that gidB mutations address many unanswered questions and explain the whole story behind phenotypic streptomycin-resistant strains exhibiting no mutation in rpsL or rrs. They also validate the hypothesis of sequential progression of resistance from low to high due to the existence of gidB alterations in the genetic background.

Keywords: drug resistance, in silico modelling, DNA sequencing, novel mutations

Introduction

Tuberculosis (TB) is a devastating infectious disease causing 1.4 million deaths, with 8 million new TB cases worldwide annually. India bears the highest TB burden, accounting for 20% of the global incidence and 65% of cases in the South-East Asian subcontinent.1 Streptomycin was the first aminoglycoside antibiotic introduced (in 1944) against Mycobacterium tuberculosis. It was initially used in monotherapy regimes, which led to the quick emergence of streptomycin resistance.2 Due to the emergence of resistance to current anti-tubercular drugs, the use of streptomycin has gained renewed interest in multidrug regimens such as directly observed therapy, short course (DOTS).3

Aminoglycosides inhibit protein translation by binding the A site of the 30S ribosomal subunit at the major groove of a triple adenine pocket in helix 44 of the 16S rRNA at positions A1408, A1492 and A1493, inhibiting tRNA translocation (Escherichia coli numbering).4 Streptomycin is slightly different, having additional interactions with the 530 loop of the 16S rRNA (rrs) and the ribosomal S12 protein (rpsL).5 Streptomycin binding with the ribosome not only inhibits tRNA translocation, but also results in accumulation of toxic protein intermediates in the cytoplasm (thus poisoning the cell), produced due to miscoding of mRNA–tRNA pairs.6 High-level streptomycin resistance has often been linked with point mutations involving the ribosomal protein S12 (rpsL gene) and mutations in the 530 loop of the 16S rRNA (rrs), mostly A514C or C517T alleles (formerly annotated as A513C and 33 C516T).7,8 However, up to 50% of clinical streptomycin-resistant isolates may present no mutation in either of these genes, and thus there is uncertainty about the resistance mechanism.
In recent times it has been inconsistently observed that mutations in gidB loci are responsible for low-level streptomycin resistance through comparative streptomycin-resistant versus -susceptible isolate genomic sequencing and the resistance profile exhibited by the knockout.\textsuperscript{9} gidB (Rv3919c) encodes an S-adenosylmethionine (SAM)-dependent 7-methylguanosine (m7G) methyltransferase that is thought to methylate the G527 in the 530 loop of the 16S rRNA. The gidB methylation site G527 lies within a hairpin loop (530), which is a mutational hotspot imparting streptomycin resistance.\textsuperscript{10–12} Ribosomal protein S12 binding is in close proximity to the hairpin loop (530) and plays an indispensable role in protein translational fidelity.\textsuperscript{11,13} Analysis of the 16S rRNA by HPLC showed that the gidB mutant lacked an m7G modification.\textsuperscript{9}

Most interestingly, alterations in gidB have been found in almost a third of low-level streptomycin-resistant clinical isolates surveyed, and they arise by spontaneous mutation at a relatively high frequency (2.8 × 10^{-6}).\textsuperscript{9} It is also noteworthy that gidB mutations were present only in isolates without rpsL and rrs mutations, all from genetic cluster Q1.\textsuperscript{15} Moreover, gidB mutant strains were 10\textsuperscript{3} times more likely to develop rrs or rpsL mutational high-level streptomycin resistance; thus, this phenomenon may have contributed to the initial failure of streptomycin as an anti-tuberculosis drug.\textsuperscript{9}

As no in vitro activity assay has yet been formulated due to substrate complexity and previous reports showing the presence of gidB mutations in both streptomycin-susceptible and resistant strains, we employed in silico methods to assess the importance of the sense mutations found and also the structure of gidB homologue with SAM has been worked out in E. coli, which has 74% homology with M. tuberculosis gidB.\textsuperscript{15}

Materials and methods

Sampling and bacterial strains

This multicentre study was conducted at Department of Microbiology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India. The genotypic protocols, i.e. DNA amplification and sequencing, were performed at the Department of Biotechnology, National Centre for Disease Control, New Delhi, India and National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra, India. All subjects included in the study were recruited after having given informed written consent. The consent proforma was duly approved by an institutional ethics review board (known as the Ethical Clearance Committee in our institute) and the protocol we used followed the approved guidelines. All subjects enrolled were anonymized. During the study, a total of 75 isolates were selected from clinical strains collected between 2007 and 2011 from patients visiting the DOTS centre and the outpatient department. All isolates were characterized as M. tuberculosis by a commercial probe-based technique (Accuprobe; Gen-Probe Inc., San Diego, CA, USA).

Drug susceptibility testing

Drud susceptibility was tested by the Lowenstein–Jensen MIC method using streptomycin concentrations of 16, 32, 64, 128 and 256 mg/L, with wild-type reference strain H37Rv. The method was as described previously.\textsuperscript{15} The peak serum concentration reaches ~30 mg/kg ~1–4 h after injection, thus isolates having MICs ≥ 32 mg/L were completely unaffected by the current therapy.\textsuperscript{17} Mutations in gidB have been found in both treatable and untreatable strains.\textsuperscript{18} Based on this finding, we evaluated the presence of bacterial growth at streptomycin MICs ≥ 32 mg/L as a breakpoint for high-level streptomycin resistance.

DNA extraction and targeted amplification

DNA from isolates in the log phase of growth was extracted using a Qiagen DNA extraction kit (Qiagen, Chatsworth, CA, USA) following the manufacturer’s instructions. A 675 bp fragment containing the entire gidB gene was amplified by a modified protocol described previously.\textsuperscript{19} The primers 5’-GTCCCTCCACTCAGCCATC-3’ (forward) and 5’-GCGGAGTGCGTAATGTCTC-3’ (reverse) were used. Amplification was performed by a PCR consisting of an initial denaturation step of 2 min followed by 35 thermal cycles of 1 min at 94°C for denaturation, 1 min at 58°C for annealing and 2 min at 72°C for extension. rpsL and rrs fragments of 505 and 1140 bp, respectively, were also amplified and sequenced. After the final cycle, samples were maintained at 4°C until further analysis. PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide and visualized by UV transillumination.

Sequencing

Following purification of PCR products and sequencing reactions with a BigDye terminator kit (ABI Prism), the sequencing products were loaded into an ABI Prism 3130xl automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA, USA). The sequences thus generated were aligned and compared with the wild-type sequence of H37Rv using ClustalW (www.ebi.ac.uk) algorithm.

Nucleotide sequence accession numbers

The sequences with alterations in the gidB region were deposited in GenBank under accession numbers HQ611141, HQ611142, HQ611143, HQ611144, HQ611145, HQ611146 and HQ611147.

Multiple sequence analysis (MSA)

Orthologous proteins from other species of mycobacteria and human were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) and the same server was employed for multiple sequence alignments by ClustalW (www.ebi.ac.uk). We used the following combined set of 12 organisms: M. tuberculosis, Mycobacterium bovis, Mycobacterium avium, Mycobacterium leprae, Mycobacterium microti, Mycobacterium ulcerans, Mycobacterium paratuberculosis, Mycobacterium gilvum, Mycobacterium vanbaalenii, Mycobacterium abscessus, Mycobacterium smegmatis and E. coli. The generated sequences were mapped and aligned using ClustalW (www.ebi.ac.uk).

Protein modelling

I-TASSER

To predict the structure of all target proteins, automated servers for structure predictions were employed that are capable of aligning unknown sequence with structure rather than sequence–sequence alignment by using complex structure-based energy scores. The I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER) was employed for the modeling of all proteins.\textsuperscript{20}

Visual Molecular Dynamics (VMD)

Structures were further analysed using VMD software (University of Illinois) and were selected on the basis of root mean square deviation (RMSD) values (<4) and <1% deviation from the Ramachandran plot.\textsuperscript{21}
**Docker analysis**

**PatchDock**
Selected structures were molecularly docked with SAM (structure obtained from www.drugbank.ca) for in silico interaction studies by submitting the structures to the PatchDock server (http://bioinfo3d.cs.tau.ac.il/ PatchDock/), which is based on shape complementarity principles.\(^{22}\)

**FireDock**
The PatchDock results were resubmitted for refinement using the FireDock server (http://bioinfo3d.cs.tau.ac.il/FireDock/), which rearranges the interface side chains and adjusts the relative orientation of the molecules.\(^{23,24}\)

**Results**
All 75 strains of *M. tuberculosis* studied were confirmed by the specific primer amplification of *gidB*, *rrs* and *rpsL* (data not shown). Out of 75 isolates studied, 52 (69%) were streptomycin resistant and 23 (31%) were streptomycin susceptible irrespective of other drugs. The relationship between MICs of streptomycin for 75 strains and the genetic alterations within the *gidB* and *rpsL* genes are shown in Table 1.

### Sequencing outcome

#### *gidB*

In *gidB*, 42/52 (80.7%) resistant isolates showed nucleotide substitutions. To the best of our knowledge, the alterations observed at codons 70 (Arg→Ser), 76 (Gly→Cys), 89 (Val→Met), 108 (Leu→Arg), 146 (Thr→Met), 149 (Ser→Arg), 160 (Leu→His), 171 (Val→Ile) and 187 (Arg→Met) have not been reported before (Table 2). We also noticed a substitution at codon 16 (CTT→CGT; Leu→Arg). Four new silent mutations at codons 110 (GTG→TTT), 141 (GGC→GCA), 175 (CGG→CGT) and 205 (GCA→GCG) were found, the alteration at codon 205 was detected in 10/52 isolates. One change, at codon 100 (TCT→TAT; Ser→Phe), was present in every subject studied.

#### Genotyping of *rpsL* and *rrs*

Alteration in *rpsL* was found in 17/52 (32.6%) resistant isolates at position 43 (AAG→AGG; Lys→Arg) and 10/52 (19.2%) at position 88 (AAG→AGG; Lys→Arg). The MICs of streptomycin for all these strains containing a point mutation were ≥128 mg/L, except for one streptomycin-resistant strain with a mutation at codon 88 (Lys→Arg), for which the MIC was 64 mg/L. A silent mutation at codon 121 with or without a mutation at codon 43 or 88 was detected in 30 (57.6%) resistant isolates. One novel silent mutation at codon 123 was also found. Also, a 1140 bp *rrs* locus amplified co-existing with a change at codon 43 in the *rpsL* gene. Notably, *gidB* mutations were accompanied to that of *rrs* mutations (30.7%; 23/75), and triple (*gidB* *rpsL* and *rrs*) mutations were accompanied at a frequency of 20% (15/75) among isolates classified as resistant. The frequency of *gidB* mutations was comparable to that of *rrs* mutations (30.7%; 23/75), and even double (*gidB* *rpsL* or *gidB* *rrs*) and triple (*gidB* *rpsL* and *rrs*) mutations were detected at a frequency of 10.7% (8/75) (Table 1). All three 516 (C→T) *rrs* mutations were accompanied by sense mutations in *gidB*.

### MSA

MSA significantly showed the presence of all the mutations in the conserved region (Figure 1). It also demonstrated that the

---

**Table 1.** Relationship between MICs of streptomycin for 75 strains and genetic alterations within the *gidB*, *rpsL* and *rrs* genes

<table>
<thead>
<tr>
<th>MIC (mg/L)</th>
<th>Susceptibility</th>
<th>No. of isolates</th>
<th>Mutations only in <em>rpsL</em></th>
<th>Mutations only in <em>gidB</em></th>
<th>Mutations in <em>rrs</em> and <em>gidB</em></th>
<th>No mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;32</td>
<td>S</td>
<td>23</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>32</td>
<td>R</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>64</td>
<td>R</td>
<td>16</td>
<td>1</td>
<td>8</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>128</td>
<td>R</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>256</td>
<td>R</td>
<td>24</td>
<td>18</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

R, resistant; S, susceptible.

\(^a\)Silent mutations not included.

\(^b\)Spontaneous change at codon 100 (Ser→Phe) not included.

---

**Table 2.** Distribution of mutations found in *gidB* in streptomycin-resistant *M. tuberculosis* isolates

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Protein change</th>
<th>Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>CTT→CGT</td>
<td>Leu→Arg (R)</td>
<td>reported</td>
</tr>
<tr>
<td>70</td>
<td>AGC→CGC</td>
<td>Arg (R)→Ser (S)</td>
<td>novel</td>
</tr>
<tr>
<td>76</td>
<td>GGC→TGC</td>
<td>Gly (G)→Cys (C)</td>
<td>novel</td>
</tr>
<tr>
<td>89</td>
<td>GTG→ATG</td>
<td>Val (V)→Met (M)</td>
<td>novel</td>
</tr>
<tr>
<td>92</td>
<td>GAA→GAC</td>
<td>Glu (E)→Asp (D)</td>
<td>reported</td>
</tr>
<tr>
<td>108</td>
<td>CTG→CGG</td>
<td>Leu (L)→Arg (R)</td>
<td>novel</td>
</tr>
<tr>
<td>146</td>
<td>AGC→ATG</td>
<td>Thr (T)→Met (M)</td>
<td>novel</td>
</tr>
<tr>
<td>149</td>
<td>AGC→CGC</td>
<td>Ser (S)→Arg (R)</td>
<td>novel</td>
</tr>
<tr>
<td>160</td>
<td>CTC→CAC</td>
<td>Leu (L)→His (H)</td>
<td>novel</td>
</tr>
<tr>
<td>171</td>
<td>GTA→ATA</td>
<td>Val (V)→Ile (I)</td>
<td>novel</td>
</tr>
<tr>
<td>187</td>
<td>AGG→ATG</td>
<td>Arg (R)→Met (M)</td>
<td>novel</td>
</tr>
<tr>
<td>100(^a)</td>
<td>TCT→TTT</td>
<td>Ser (S)→Phe (F)</td>
<td>reported</td>
</tr>
<tr>
<td>110</td>
<td>GTG→GTT</td>
<td>silent</td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>GGC→GCA</td>
<td>silent</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>CGG→CGT</td>
<td>silent</td>
<td></td>
</tr>
<tr>
<td>205</td>
<td>GCA→GCG</td>
<td>silent</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Spontaneous change in wild-type strain.
presence of the omnipresent change, i.e. that at codon 100, was actually a spontaneous polymorphism in the H37Rv reference strain.

**Protein modelling and docking analysis**

gidB (Rv3919c) and its mutants were modelled using the I-TASSER server. The modelled proteins had <1% disagreement with the Ramachandran plot in VMD software. The FireDock results showed global energy $-37.74$, attractive/repulsive van der Waals energy $-16.65/6.50$ and atomic contact energy $-12.54$. The locations of the studied amino acids are shown in Figure 2. The wild-type strain docking with SAM is shown in Figure 3(a and b).

**Structural variation found in gidB mutated sites**

- **Codon 160** This belongs to the β5 chain and this change affects adjacent β4 (amino acid residues 130–138) and subsequently changes the cavity structure and alignment of the nearest interacting amino acid residue 137-Arg (Figure 3c).
- **Codon 100** This mutation belongs to α3 and this change does not disrupt the helix structure (Figure 3d).
- **Codon 16** This belongs to α1, which aligns with hydrophobic loop 2; thus, alignment changes were observed at interacting amino acid residues 70-Ser and 74-Leu, thus changing binding cavity morphology (Figure 3e).
- **Codon 108** This belongs to α3 and this affects adjacent motif β4 and subsequently changes were observed at 137-Arg and 138-Ala,
thereby changing the alignment and nature of the cavity. Also, α3 interacts with hydrophobic loop 2, thus changing the alignment of interacting amino acid residues 70 and 74 (Figure 3f).

**Codon 70** This mutation changes the interacting amino acid residue 70-Ser, which forms hydrogen bonds with the ribose sugar of SAM, the change to Arg, which has a bulkier side chain, changes the cavity to the greatest extent (Figure 3g).

**Codons 149 and 171** The codon 171-Val→Ile mutation does not affect the structure as the residues are similar in nature and size and lie away from the binding pocket, while the codon 149-Ser→Arg change is in α4 and lies close to 137-Arg, which is the scaffold for SAM binding; this change drastically changes the binding pocket, affecting 70-Ser and 74-Leu alignments as well (Figure 3h).

**Codon 76** Though this codon is close to interacting amino acid residues 71, 72, 73 and 74 and the hydrophilic amino acid residue was replaced with a hydrophobic amino acid residue, there was no change in the docking site. This is due to the fact that Gly-76 does not interact with SAM and Cys is a small amino acid residue, which aligns similarly to Gly (Figure 3i).

**Codon 89** This lies in the β2 chain, but is exposed away from the core of the structure, so there is not much change in the structure due to this mutation, though it is close to interacting amino acid residues 91–94 (Figure 3j).

**Codon 146** This lies in the α4 region and is close to the interacting amino acid residues 137 and 138. This mutation has an overall impact on the structure of the binding cavity, α4 connects β4 and β5 in a tight twist (150–152 3_10 helix); thus, alteration in α4 changes both β4 and β5, giving rise to an overall change in structure (Figure 3k).

**Codon 187** This lies in β6 of wild-type. Arg→Met conversion destabilizes the β6 of the mutant and the resulting structure cannot form a β6 beta plate structure, thus destabilizing the 7-mer β core of gidB. Almost all interacting amino acid residues were distorted in the resulting structure (Figure 3l).

**Discussion**

The growing burden of drug-resistant strains of *M. tuberculosis* demands a greater understanding of the genetic basis and molecular mechanisms of the development of resistance to drugs used for...
Co-occurrence of mutants than in wild-type cells.

The gidB gene is highly conserved in both Gram-positive and Gram-negative bacteria and is found in all bacterial genomes sequenced to date, including that of Mycoplasma genitalium, which has the smallest genome among all known self-sustaining living organisms. Therefore, it was assumed that gidB mutations may cause low-level streptomycin resistance in virtually all bacteria.

The mutations detected were all at either codon 43 (Lys → Arg) or codon 117 (Thr → Leu). During alignment after BLAST analysis, we observed a nucleotide substitution at codon 100 (Ser → Phe), which was observed in each subject studied, with or without other alterations in the target region. This was clarified by application of the mutation mapping protocol MSA, which showed that the substitution was a spontaneous alteration in the wild-type H37Rv strain (Figure 1). MSA also strongly supported the significance of various mutations in the conserved gidB region shown earlier by bioinformatics analysis.

Our results showed that most of the resistant strains for which the MICs were high (≥ 256 mg/L) had an alteration in the rpsL gene. The mutations detected were all at either codon 43 (Lys → Arg) or codon 88 (Lys → Arg). This is in agreement with published data. Three out of five isolates for which the MICs were high (≥ 128 mg/L) did not show any mutation in the rpsL gene, but had alterations in the rs locus.

One study found that most gidB mutations were associated with isolates for which the MIC was ≤ 64 mg/L, without having any mutation in rpsL. But as per our study outcome, we can speculate that these isolates might be more prone to mutate rs/rpsL than the isolates that do not have gidB mutations.
is scope for further investigation of how a mutation at this locus leads to the development of streptomycin resistance. However, occurrence of simultaneous mutations in two different gene loci, which has low probability, suggests that the acquisition of such mutations confers an adaptive advantage. The gidB mutant strains could be used as models for screening new aminoglycoside derivatives; this screening may be more effective in rpsL and gidB mutants as the site that is modified is the same and the resistance level is different for different aminoglycosides. Our study will enhance our understanding of the molecular mechanisms of drug resistance.

Additionally, the present results should assist in the process of designing new molecular probes, which promise more rapid detection rates. Further characterization is required for all mutants of gidB to ascertain the extent of changes in methylation activity.

Acknowledgements
We acknowledge the unconditional support of Dr D. S. Rawat and his scientific input during this study.

Funding
This work was supported by the Indian Council of Medical Research (Senior Research Fellowship No. 80/602/2008-ECD-1 to J. S. V.).

Transparency declarations
None to declare.

References