Mutations in \( \text{gidB} \) Confer Low-Level Streptomycin Resistance in \( \text{Mycobacterium tuberculosis} \)\(^\dagger\)

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The global threat posed by drug-resistant strains of \( \text{Mycobacterium tuberculosis} \) demands a greater understanding of the genetic basis and molecular mechanisms that govern how such strains develop resistance against various antituberculous drugs. In this report, we examine a new genetic basis for resistance to one of the oldest and most widely used second-line drugs employed in tuberculosis therapy, streptomycin (SM). This marker for SM resistance was first discovered on the basis of genomic data obtained from drug-resistant \( \text{M. tuberculosis} \) strains collected in Japan, wherein an association was observed between SM resistance and a mutation in \( \text{gidB} \), a putative 16S rRNA methyltransferase. By evaluating an isogenic \( \Delta \text{gidB} \) mutant strain constructed from strain H37Rv, we demonstrate the causal role of \( \text{gidB} \) in conferring a low-level SM-resistant phenotype in \( \text{M. tuberculosis} \) with a 16-fold increase in the MIC over the parent strain. Among clinical isolates, the modest increase in SM resistance conferred by a \( \text{gidB} \) mutation leads to an MIC distribution of \( \text{gidB} \) mutation-containing strains that spans the recommended SM breakpoint concentration currently used in drug susceptibility testing protocols. As such, some \( \text{gidB} \) mutation-containing isolates are found to be SM sensitive, while others are SM resistant. On the basis of a pharmacodynamic analysis and Monte Carlo simulation, those isolates that are found to be SM sensitive should still respond favorably to SM treatment, while nearly half of those found to be SM resistant will likely respond poorly. This report provides the first microbiological evidence for the contribution of \( \text{gidB} \) in streptomycin resistance and examines the clinical implications of mutations in the \( \text{gidB} \) gene.

The World Health Organization (WHO) 2010 report estimates that 440,000 cases of multidrug-resistant (MDR) tuberculosis (TB) emerged in 2008, with 5.4% of these cases being extensively drug resistant (XDR) TB (36). The strains responsible for producing these forms of TB incur far greater mortality and morbidity to TB patients than drug-sensitive strains (12, 13). If left undetected, these drug-resistant strains can further propagate throughout a community with the potential to cause an epidemic of untreatable, drug-resistant TB. Yet if the patients receive a proper diagnosis and are properly cared for, the success of treatment of MDR-TB can be as high as 85% (36). Therefore, there is an urgent need for faster, more accurate diagnostic alternatives to the current, culture-based drug susceptibility testing protocols. As such, some \( \text{gidB} \) mutation-containing isolates are found to be SM sensitive, while others are SM resistant. On the basis of a pharmacodynamic analysis and Monte Carlo simulation, those isolates that are found to be SM sensitive should still respond favorably to SM treatment, while nearly half of those found to be SM resistant will likely respond poorly. This report provides the first microbiological evidence for the contribution of \( \text{gidB} \) in streptomycin resistance and examines the clinical implications of mutations in the \( \text{gidB} \) gene.

In many parts of the world, streptomycin (SM) is the first of the second-line, aminoglycoside drugs used to treat TB treatment failures. Patients who fail their first course of front-line therapy are given a retreatment regimen recommended by the WHO that adds SM to the three front-line agents isoniazid, rifampin, and ethambutol for 8 months (35). The primary mode of action through which SM exerts its toxic effects involves binding to the 16S rRNA and disrupting protein translation (9, 28). Mutations in the \( \text{rrs} \) gene, which encodes the 16S rRNA, in either the 530 stem-loop or 915 nucleoside regions, both of which are situated close to the SM-binding site, confer moderate levels of SM resistance. A mutation at lysine residue 42 or lysine residue 87 of the S12 ribosomal protein, which interacts intimately with the 16S RNA near the SM-binding site, confers high-level resistance to the drug (3, 7, 15, 31). Approximately 85% of SM-resistant \( \text{Mycobacterium tuberculosis} \) isolates observed throughout the world contain one of these genetic markers, leaving roughly 15% of SM-resistant isolates with a yet-to-be-discovered marker for resistance (29, 32).

Recently, we and others have reported an association between \( \text{M. tuberculosis} \) clinical isolates harboring a variety of mutations in the \( \text{gidB} \) gene (Rx3919e) and a low-level SM-resistant phenotype (22, 30, 33). However, in all reports, mutations were present in both SM-sensitive and SM-resistant isolates. In this study, we sought to determine definitively the role of \( \text{gidB} \) in conferring SM resistance in \( \text{M. tuberculosis} \). A knockout mutant constructed from strain H37Rv exhibited a low-level SM resistance phenotype and a growth rate comparable to that of the parent strain. We further examined the

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† Supplemental material for this article may be found at http://aac.asm.org.

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The ΔgidB mutant was generated following a previously reported protocol (24). Successful gene disruption was confirmed by direct sequencing and PCR using primers within and flanking the deleted region. For complementation of ΔgidB, PCR-amplified gidB was cloned into pMV261 at the HindIII and BamHI sites (10). Successful transformants were selected on Middlebrook 7H11 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase, 0.5% glycerol, and 50 μg/ml hygromycin.

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>Laboratory strain of <em>M. tuberculosis</em></td>
<td>ATCC 27294</td>
</tr>
<tr>
<td>ΔgidB</td>
<td>In-frame ΔgidB mutant of H37Rv</td>
<td>This work</td>
</tr>
<tr>
<td>ΔgidB pmMVgidB</td>
<td>ΔgidB mutant complemented with pmMV261-gidB</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Plasmids**

- pCR2.1: Cloning vector, Km<sup>+</sup> Amp<sup>+</sup> Invitrogen
- p2NIL: Cloning vector, Km<sup>+</sup> 24 Invitrogen
- pm261: Complementation vector, Hyg<sup>+</sup> 10 Invitrogen
- pm261-gidB: Complementing construct containing gidB cloned into pmMV261 This work
- pGOAL17: Plasmid carrying sucB and lacZ genes on a PacI cassette, Amp<sup>+</sup> 24 Invitrogen
- p2NILgidBKO: p2NIL vector carrying upstream and downstream regions of gidB, PacI cassette from pGOAL17, Km<sup>+</sup> 24 Invitrogen

Clinical implications of a gidB mutation and show that the MIC distribution of a population of gidB mutation-containing clinical isolates straddles the recommended breakpoint concentration of SM, resulting in some gidB mutation-containing isolates being classified as SM sensitive and others being classified as SM resistant. Finally, we present a pharmacodynamic Monte Carlo simulation to address the efficacy of SM therapy on those gidB mutation-containing clinical isolates that are classified as SM sensitive.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, gidB-knockout mutant generation, and complementation of knockout mutant.** The laboratory strains, plasmids, and primers used in this study are summarized in Tables 1 and 2. One Shot Top10 *Escherichia coli* (Invitrogen) cells were used for all cloning. *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 broth (Becton Dickinson, NJ) supplemented with 10% albumin-dextrose-catalase, 0.05% Tween 80, and 0.2% glycerol. All antibiotics used in this study were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations: kanamycin at 50 μg/ml for *M. tuberculosis*. The ΔgidB mutant strain was generated from H37Rv by homologous recombination. Upstream and downstream regions flanking gidB were amplified by PCR and cloned into p2NIL at the HindIII and NotI sites (24). The lacZ and sucB-containing PacI fragment from pGOAL17 was cloned into the PacI site of p2NIL. The ΔgidB mutant was generated following a previously reported protocol (24). Successful gene disruption was confirmed by direct sequencing and PCR using primers within and flanking the deleted region. For complementation of ΔgidB, PCR-amplified gidB was cloned into pMV261 at the HindIII and BamHI sites (10). Successful transformants were selected on Middlebrook 7H11 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase, 0.5% glycerol, and 50 μg/ml hygromycin.

**RNA extraction and quantitative real-time PCR analysis.** Three independent biological cultures of H37Rv and ΔgidB were grown in supplemented Middlebrook 7H9 medium to optical densities at 650 nm of 0.25 (early log stage), 1.0 (log stage), and 2.8 (late log stage). Cells were harvested by centrifugation, and the pellets resuspended in 900 μl Trizol reagent (Invitrogen). The cell suspension was transferred to a 1.5-ml screw-cap microtube containing approximately 0.2 ml of 0.1-mm zirconium/silica beads (Biospec Products, Bartlesville, OK). Cells were then disrupted by three 30-s pulses at a speed setting of 6 on a Roche MagNA Lyser bead beater and with a 5-min ice incubation after each pulse. Samples were centrifuged at 9,500 × g for 45 s, and the supernatants were transferred to a fresh microcentrifuge tube. A 1/10 volume of 1-bromo-3-chloro-4-iodophenol (Molecular Research Center, Cincinnati, OH) was added to the supernatant, the tube was inverted by hand, and the mixtures were left to separate on ice for 10 min. The samples were centrifuged at 9,500 × g for 10 min at room temperature. The supernatant was transferred to a fresh microcentrifugation tube, an equal volume of 70% ethanol was added, and the mixture was inverted by hand. The solution was applied onto Qiagen RNasey minicolumns, and the RNA was purified according to the manufacturer’s protocol, including that for the on-column DNase digestion.

First-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qRT-PCR) was performed using TaqMan Universal PCR master mix with AmpErase uracil-N-glycosylase reagents (Applied Biosystems, Carlsbad, CA). Primer and probe sequences are given in Table 2. The absolute quantitation method was employed, where a standard curve ranging from 1.5 pg to 15 ng genomic DNA was used for quantification for each primer-probe set. Quantified mRNA levels for gidB, par4, and parB were normalized to the quantified mRNA levels of the housekeeping gene *rpsL*. Each RNA sample was independently reverse transcribed and tested in duplicate for each primer-probe set. Control samples with no reverse transcriptase added during cDNA synthesis were also evaluated in duplicate for all samples to confirm the absence of DNA contamination.

**Clinical strains.** Characteristics of the patients from whom clinical isolates were obtained are detailed elsewhere (11, 33). Eighteen gidB mutation-containing isolates and 14 gidB mutation-free isolates were evaluated in this study. Strains were cultured in supplemented Middlebrook 7H9 medium. The resistance and mutation profiles of the clinical isolates used in this study are detailed in Table 3. Strain H37Rv and four clinical isolates containing the SM resistance-associated mutation *rpsL*. A128G (K42R) were used as controls.

**Drug susceptibility testing.** The MIC values of strain H37Rv and ΔgidB isolates were determined using the broth microdilution method. Strains were grown in supplemented Middlebrook 7H9 medium to an optical density at 650 nm of 0.25 and subsequently diluted with supplemented Middlebrook 7H9 to prepare a 10<sup>−3</sup> dilution for use in the assay. Each drug of interest was added to the first column of a round-bottom, 96-well plate in triplicate. A 2-fold serial dilution of

**TABLE 2. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Purpose and oligonucleotide</th>
<th>Forward primer sequence (5’→3’)</th>
<th>Reverse primer sequence (5’→3’)</th>
<th>Probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construction of mutant strain gidB upstream region</td>
<td>GGATCCGCGCGTCGTCGAGCTCTG</td>
<td>AGCCGCGCCTTTGTTCCCGCGCTCC</td>
<td></td>
</tr>
<tr>
<td>gidB downstream region</td>
<td>AAGGCTGCTGGGACCCTCGTGG</td>
<td>GGGATCCCTTACACGAAGATACGGGAG</td>
<td></td>
</tr>
<tr>
<td>gidB</td>
<td>AACCGTTCACCGGCTTCCTACTG</td>
<td>GGGATCCATGCTTCCACGGC</td>
<td></td>
</tr>
<tr>
<td>qRT-PCR analysis</td>
<td>AATTGGAGGATGCGGCTGTTGATAC</td>
<td>GAGGCCGCGTTCGCTTT</td>
<td>CCAACGCGGCAATT</td>
</tr>
<tr>
<td>par4</td>
<td>AAGGCGGGCTGGGACATG</td>
<td>CCCGCTGGCGGAA</td>
<td>CACGACGCTCAAG</td>
</tr>
<tr>
<td>parB</td>
<td>TCATTGGCTGGATAC</td>
<td>CCCGCTCCCATCACTATC</td>
<td>CGGGTGCGCTAC</td>
</tr>
</tbody>
</table>

**Figure 1.** Western blot analysis of ΔgidB mutant strain. Lane 1, wild-type H37Rv; lane 2, ΔgidB mutant. Western blot analysis of ΔgidB mutant strain. Lane 1, wild-type H37Rv; lane 2, ΔgidB mutant.
the drug was created down the remaining columns with supplemented Middlebrook 7H9 medium as the diluent. An equal volume of the 1000-fold-diluted cell suspension was added to each drug-containing well for a final volume in each well of 100 µl. Plates were incubated at 37°C for 14 days, after which cell growth was visually inspected. The MIC value was defined as the lowest drug concentration of 100 µl that resulted in >1% growth compared to the growth in no-drug control wells. Due to the prevalence of these single nucleotide polymorphisms among the clinical isolates, they were not considered to be associated with streptomycin resistance.

The MIC values of all clinical isolates were determined using the proportion method (4). The MIC values for the 100-fold-diluted cell suspension were determined using the proportion method (4). The MIC values for drugs with single nucleotide polymorphisms in the rpsL and gidB genes were determined using the proportion method (4). The MIC values for drugs with single nucleotide polymorphisms in the rpsL and gidB genes were determined using the proportion method (4).

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evaporation of the inoculum. After 24 h, the screw caps were tightened. Slants were read for MIC determination after 40 days of growth. The MIC value in both media was defined as the lowest drug concentration that resulted in <1% growth compared to the growth on no-drug control plates/slants.

Lowenstein-Jensen medium and Middlebrook 7H10 agar medium. Streptomycin was incorporated into the LJ medium prior to inspissation and dispensed in 10-ml volumes into screw-cap bottles (28 ml). The media were inspissated at 85°C for 45 min. All drug-loaded media were stored at 4°C and used within 2 weeks. Streptomycin-loaded 7H10 agar plates were prepared according to NCCLS guidelines. Plates were stored at 4°C and used within 1 week.

Statistical analysis. The MIC distributions of the gidB mutation-containing and gidB mutation-free clinical isolates were evaluated for normality using the D’Agostino-Pearson test. The normality test, F-test comparison of variances, and t-test comparison of the means were performed using either Microsoft Excel or GraphPad Prism (version 5) software.

Monte Carlo simulation. The probability that strains classified as either SM sensitive or SM resistant would respond to SM therapy was estimated using a Monte Carlo simulation. The algorithm entailed computationally choosing an MIC value from the distribution of MICs obtained for the gidB mutation-containing isolates. Each value was chosen with a normally distributed probability. The maximum concentrations in serum (C\text{max}) were also computationally chosen with a normally distributed probability based on C\text{max} data obtained from a population of TB patients who received an intravenous dosage of 12 to 15 mg SM/kg of body weight (39). The mean C\text{max} and standard deviation values for this population of patients were obtained through personal communication with Chuck Peloquin. The C\text{max}/MIC value was subsequently calculated and monitored if the value exceeded 10. The process of computationally choosing an MIC and C\text{max} and calculating the corresponding C\text{max}/MIC value was performed 10,000 times for the gidB mutation-containing isolates to generate a distribution of C\text{max}/MIC values and to calculate the probability of exceeding a C\text{max}/MIC value of 10. The same recursive algorithm was performed for the gidB mutation-free isolates. Microsoft Excel software was employed for the Monte Carlo simulation using the NORMINV(rand(),mean,stdev) function.

RESULTS

Growth rate comparison. To assess the importance of gidB mutations in conferring SM resistance, we constructed an isogenic deletion mutant by homologous recombination in laboratory strain H37Rv. Successful construction of this deletion was confirmed by direct sequencing and PCR using primers within and flanking the deleted region (data not shown). To evaluate the potential fitness cost associated with a gidB deletion, we quantified the number of colonies formed as a function of time. We observed comparable growth rates between the two strains, shown in Fig. 1, indicating no apparent cost in overall fitness associated with the gene deletion.

Expression profile of gidB, parA, and parB. By simple inspection of the genetic arrangement of gidB within the M. tuberculosis chromosome, one might expect its deletion to induce polar effects; as it seemingly belongs to an operon with the critical, chromosomal partitioning genes, parA and parB (see Fig. S1 in the supplemental material). Casart et al. had previously reported coexpression of gidB with parA and parB with parB in Mycobacterium bovis BCG, a slow-growing mycobacterium closely related to M. tuberculosis whose genetic arrangement of gidB, parA, and parB is nearly identical to that of M. tuberculosis (6). However, their study also showed the presence of at least one distinct transcription start site upstream of each of the three genes, suggesting that their respective transcription profiles may be independent of one another to a certain extent. To determine whether polar effects may have been induced by a gidB deletion, the expression profiles of parA, parB, and gidB were compared between the mutant and parent strains at the early log, log, and late log stages of growth using molecular beacons and qRT-PCR to measure transcript levels. As shown in Fig. 2, expression levels of parA and parB were not affected by the deletion of the upstream gidB gene, consistent with the comparable growth rates observed between mutant and parent strains.

Drug susceptibility testing. The drug susceptibility profiles of the ΔgidB mutant and H37Rv parent strains are shown in Fig. 3. The streptomycin MIC of the ΔgidB mutant was 16-fold higher than that of the H37Rv parent strain, resulting in a low-level SM-resistant phenotype. Complementation of the ΔgidB mutant with a wild-type copy of gidB restored MIC levels back to near wild-type levels. To evaluate for cross-resistance conferred by the gidB deletion, drug susceptibility testing was also performed on other drugs that interact directly
DSTs, however, two (18, 34). Due to no growth or contamination in the 7H10 agar to replicate the DST conditions in use throughout the world media following NCCLS- and WHO-recommended protocols isolates. MIC levels were determined in both 7H10 agar and LJ other drugs.

with the bacterial ribosome and drugs with targets other than the ribosome (Table 4). No difference in MIC levels was observed between the mutant and parent strains for any of these other drugs.

Clinical relevance of gidB mutation. Since the MIC value of the ΔgidB mutant was higher than that of parent strain H37Rv, we hypothesized that the distribution of MIC levels of a population of clinical isolates with a gidB mutation would accordingly trend higher than that of a population of isolates without a mutation in gidB. Moreover, the slight upward shift in the MIC distribution of gidB mutation-containing isolates would span the breakpoint concentration of SM specified in standard DST protocols, such that some isolates, despite harboring a gidB mutation, would have an MIC less than or equal to the breakpoint concentration and thus be classified SM sensitive, while other isolates, also harboring a gidB mutation, would have an MIC higher than the breakpoint concentration and be classified SM resistant.

To evaluate the effects of alterations in gidB in clinical isolates, we compared the aggregated MIC levels of 18 gidB mutation-containing isolates to those of 14 gidB mutation-free isolates. MIC levels were determined in both 7H10 agar and LJ media following NCCLS- and WHO-recommended protocols to replicate the DST conditions in use throughout the world (18, 34). Due to no growth or contamination in the 7H10 agar DSTs, however, two gidB mutation-free isolates and one gidB mutation-containing isolate had to be omitted from the analysis. The identities of the isolates omitted from analysis on 7H10 agar and all MIC values are provided in Table 3. The MIC levels of isolates containing a gidB mutation were, on average, 4-fold higher in both 7H10 and LJ media than their gidB mutation-free counterparts, thereby confirming our experimental results obtained with laboratory strains (Fig. 4; P = 0.00001 for 7H10 medium; P = 0.0003 for LJ medium). The clinical breakpoint concentration suggested for both methods is indicated by an arrow on both Fig. 4A and B (37). As a result of the upward shift in MIC distributions, part of the population of gidB mutation-containing isolates has an MIC less than or equal to the SM breakpoint concentration and thus was classified as SM sensitive, while the other part of the population has an MIC greater than the breakpoint concentration and thus was classified as SM resistant. These results reconcile the ambiguity surrounding the classification of some clinical isolates as SM resistant and others as SM sensitive, despite the presence of a gidB mutation in both groups.

Pharmacodynamic analysis and Monte Carlo simulation of the efficacy of streptomycin therapy for patients with gidB mutation-containing strains. Given that some strains harboring a gidB mutation will be classified as SM sensitive and patients will potentially be prescribed a course of SM therapy, we sought to explore the therapeutic efficacy of SM therapy on such strains. An initial attempt to compare clinical outcomes between patients harboring a gidB mutation-containing isolate and patients harboring a gidB mutation-free isolate was inconclusive, since most drug-resistant cases were prescribed a multidrug treatment that confounded the effects of SM therapy. As such, we adopted a pharmacodynamic approach coupled with a Monte Carlo simulation to evaluate the therapeutic efficacy of SM therapy for TB patients harboring gidB mutation-containing M. tuberculosis strains.

For aminoglycosides, which exhibit concentration-dependent killing, Cmax/MIC is the pharmacodynamic parameter that best correlates with bactericidal activity and clinical response (8, 21, 25). Although a Cmax/MIC target has not been established specifically for M. tuberculosis, a Cmax/MIC value of 10 is a generally accepted therapeutic target for the aminoglycosides (14, 16, 21). A simulation of 10,000 trials was performed to determine the distribution of calculated Cmax/MIC values for gidB mutation-containing isolates and for gidB mutation-free isolates. Each of the 10,000 Cmax s used in the calculation was computationally chosen from a distribution of clinically determined Cmax s (39). The MIC values used in this

### TABLE 4. Drugs evaluated for cross-resistance

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Drug</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA-inhibiting drugs</td>
<td>Kanamycin</td>
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</tr>
<tr>
<td></td>
<td>Amikacin</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Capreomycin</td>
<td>1.25</td>
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<td></td>
<td>Paromomycin</td>
<td>0.5</td>
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<td></td>
<td>Hygromycin</td>
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<tr>
<td></td>
<td>Geneticin</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Spectinomycin</td>
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<tr>
<td></td>
<td>Kasugamycin</td>
<td>&gt;128</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
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</tr>
<tr>
<td>23S rRNA-inhibiting drugs</td>
<td>Linezolid</td>
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</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
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<tr>
<td></td>
<td>Clindamycin</td>
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<td></td>
<td>Clarithromycin</td>
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<td></td>
<td>Thioestreton</td>
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<td>Non-ribosome-inhibiting drugs</td>
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<td></td>
<td>Ethambutol</td>
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<td>Isoniazid</td>
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</tr>
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<td></td>
<td>Clinafloxacin</td>
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</tr>
<tr>
<td></td>
<td>para-Aminosalicylic acid</td>
<td>0.0625</td>
</tr>
</tbody>
</table>
those of their were, on average, 4-fold higher in 7H10 medium and LJ medium than PSM resistant. The MIC levels of isolates containing a point concentration are classified as SM sensitive, while those with an medium). Isolates with an MIC less than or equal to the SM break-

gidB

10,000) of isolates that harbored a gidB mutation-containing clinical isolates in Middlebrook 7H10 agar (A) and Lowenstein-Jensen egg-based medium (B). Frequency is represented on a semilog plot, and the MIC values are shown in parentheses. Fourteen gidB mutation-free isolates and 18 gidB mutation-containing isolates were initially evaluated in this study. However, for 7H10 me-
dium drug susceptibility testing, two gidB mutation-free isolates and one gidB mutation-containing isolate were lost to contamination. The identity of the omitted 7H10 medium isolates and all MIC values are provided in Table 3. The recommended SM breakpoint concentration is indicated by an arrow (2 μg/ml in 7H10 medium; 4 μg/ml in LJ medium). Isolates with an MIC less than or equal to the SM break-

point concentration are classified as SM sensitive, while those with an MIC greater than the SM breakpoint concentration are classified as SM resistant. The MIC levels of isolates containing a gidB mutation were, on average, 4-fold higher in 7H10 medium and LJ medium than those of their gidB mutation-free counterparts (P = 0.00001 for 7H10 medium; P = 0.00003 for LJ medium).

FIG. 4. Distribution of MICs of gidB mutation-free and gidB mutation-containing clinical isolates in Middlebrook 7H10 agar (A) and Lowenstein-Jensen egg-based medium (B). The frequency is presented on a semilog plot, and the MIC values are shown in parentheses. The MIC distribution was determined in agar-based media (1, 16, 17).

On the basis of a 10,000-trial simulation, 74.3% (7,431/ 10,000) of isolates that harbored a gidB mutation achieved a C_max/MIC value of at least 10, compared to 99.5% (9,953/ 10,000) of isolates without a gidB mutation. Of the gidB mu-
tation-containing strains, 48.1% (4,812/10,000) had an MIC less than or equal to the SM breakpoint concentration of 2 μg/ml (i.e., SM sensitive) and 51.9% (5,188/10,000) had an MIC greater than the SM breakpoint concentration (i.e., SM resistant). Of the 48.1% SM-sensitive, gidB mutation-containing isolates, 98.5% (4,738/4,812) achieved a C_max/MIC value of at least 10 and should therefore respond favorably to SM therapy. In contrast, of the 51.9% SM-resistant, gidB mutation-containing isolates, only 51.9% (2,693/5,188) were estimated to respond favorably to SM therapy. These statistics stand in sharp contrast to those for isolates that contain no mutation in gidB. From a 10,000-trial simulation of the gidB mutation-free isolates, 99.6% (9,963/10,000) had an MIC less than or equal to the SM breakpoint concentration, with 99.6% (9,920/9,963) of those isolates achieving a C_max/MIC value of at least 10, while 89.2% (33/37) of the remaining 0.4% (37/10,000) SM-resistant, gidB mutation-free isolates achieved a C_max/MIC value of at least 10.

DISCUSSION

In recent years, a series of articles by Ochi and colleagues reported the role of GidB in conferring low-level SM resistance in E. coli, Streptomyces coelicolor, and Bacillus subtilis (19, 20, 22). The first of these reports further extended their study to clinical isolates of M. tuberculosis, whereby an associ-
ation was observed between isolates harboring a gidB mutation and a low-level SM-resistant phenotype (22). Indeed, a recent study reported by our group also revealed an association be-
tween harboring a gidB mutation and a low-level SM-resistant phenotype in clinical isolates (33). However, in both studies, some isolates harboring a gidB mutation were found to be SM sensitive, while other isolates also harboring a gidB mutation—the same mutation in some cases—were found to be SM resistant. In this study, we demonstrated by gene knockout that in M. tuberculosis, gidB definitively plays a role in conferring low-level SM resistance. Similarly, clinical isolates harboring one of a variety of gidB mutations also exhibited a low-level SM-resistant phenotype.

GidB functions as an S-adenosylmethionine-dependent, 16S rRNA methyltransferase (22). Its methylation target in E. coli is the guanine base at nucleoside position 527 of the 16S rRNA (22). The corresponding nucleoside in M. tuberculosis is G518, with which SM directly interacts and which also lies in the functionally critical, 530 stem-loop pseudoknot region of the 16S rRNA A site (5, 26). Given that most of the clinically observed gidB mutations occurred in residues that are highly conserved in both Gram-positive and Gram-negative bacteria, it is likely that GidB’s methyltransferase function is altered to some degree (see Fig. S2 in the supplemental material). We speculate that the altered methyltransferase function changes the methylation status of the 16S rRNA at position 518G, thereby interfering with SM binding and consequently produc-

ing isolates, 98.5% (33/37) of the remaining 0.4% (37/10,000) SM-resistant, gidB mutation-free isolates achieved a C_max/MIC value of at least 10.

an analysis were those determined in 7H10 medium, since previ-

ous studies that examined the correlation between pharmaco-
dynamic indices and clinical response employed MIC values that were determined in agar-based media (1, 16, 17).

On the basis of a 10,000-trial simulation, 74.3% (7,431/ 10,000) of isolates that harbored a gidB mutation achieved a C_max/MIC value of at least 10, compared to 99.5% (9,953/ 10,000) of isolates without a gidB mutation. Of the gidB mu-
tered GidB methylation site that we speculate, these known markers also reside in or interact with the 530 stem-loop region of the A site and modify the binding affinity of SM to the 16S rRNA, thereby diminishing its toxic effects (5, 9, 27).

On the basis of the results of our pharmacodynamic analysis and Monte Carlo simulation, almost half of gidB mutation-containing isolates that are determined to be SM resistant by the 7H110 medium-based DST protocol may respond poorly to SM therapy. This analysis demonstrates the power of current 7H110 medium-based DST methods to differentiate between those gidB mutation-containing strains that will respond favorably to SM therapy and those that may not. However, because the pharmacodynamic analysis and simulation were limited to the 7H110 medium-derived MIC results, it is uncertain whether the current LJ medium-based DST protocol affords the same level of differentiation. Given that LJ medium-based DSTs are still widely performed throughout the world, further clinical studies are needed to correlate the clinical outcome with pharmacodynamic indices that are based on LJ medium-derived MIC data to determine whether the current LJ medium-based DST protocol is adequate in identifying gidB mutation-containing isolates that may respond poorly to SM therapy.

In support of the need for more correlation studies between clinical outcome and LJ medium-based pharmacodynamic indices is the question of whether the breakpoint concentration of LJ medium-based DST protocols is set appropriately. In this study, all of the isolates that were classified as SM resistant in 7H110 medium were also classified as SM resistant in LJ medium. But an additional six gidB mutation-containing isolates and one gidB mutation-free isolate were classified as SM resistant in LJ medium, while they were classified as SM sensitive in 7H110 medium (Table 3). This discordance suggests that the SM breakpoint concentration in LJ medium may be set too low, resulting in the classification of some isolates as SM resistant when they are actually SM susceptible. These isolates would represent unfortunate missed opportunities for which one more drug (i.e., SM) would be erroneously considered unsuitable for use in treatment. Without studies that correlate clinical outcome with pharmacodynamic indices that are based on LJ medium-derived data, it would be misguided for us to suggest an alternative breakpoint concentration for LJ medium-based DST protocols with just the LJ medium-derived MIC data alone. The marked discordance in SM susceptibility between the 7H110 medium- and LJ medium-based DST results was also observed in our previous study (33). Research efforts are also needed to understand the basis for this seemingly SM-specific discordance in culture-based SM DST methods in order to either modify existing culture-based protocols or develop alternative ways to accurately determine the SM susceptibility profile of clinical strains.

The pharmacodynamic analysis and Monte Carlo simulation also highlight the importance of identifying those gidB mutation-containing strains that may respond poorly to SM therapy. Such isolates may account for up to 15% of the SM-resistant clinical strains for which a genetic marker had yet, until now, to be identified. Identifying such strains would preclude their potentially ineffective treatment with SM and instead direct the use of other drugs to which gidB mutation-containing strains are still susceptible (e.g., kanamycin, amikacin, and capreomycin) (Table 4).

As DSTs move from culture-based methods toward molecular-based diagnostics and because clinical strains may harbor a variety of mutations in gidB, the challenge in developing means to quickly and accurately differentiate strains that harbor one of many mutations in a given gene remains. Our results suggest that for gidB this may remain problematic both because the number of mutations required to accurately assess gidB status is large and also because the impact of specific mutations in gidB on the resistance level of the isolate remains unclear. Thus, at least for this gene, phenotypic DST will for the moment remain the most reliable way of predicting therapeutic response to treatment with streptomycin.

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