Novel \textit{katG} mutations causing isoniazid resistance in clinical \textit{M. tuberculosis} isolates

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We report the discovery and confirmation of 23 novel mutations with previously undocumented role in isoniazid (INH) drug resistance, in catalase-peroxidase (\textit{katG}) gene of \textit{Mycobacterium tuberculosis} (\textit{Mtb}) isolates. With these mutations, a synonymous mutation in \textit{fabG1}\textsuperscript{1,6,7}, and two canonical mutations, we were able to explain 98\% of the phenotypic resistance observed in 366 clinical \textit{Mtb} isolates collected from four high tuberculosis (TB)-burden countries: India, Moldova, Philippines, and South Africa. We conducted overlapping targeted and whole-genome sequencing for variant discovery in all clinical isolates with a variety of INH-resistant phenotypes. Our analysis showed that just two canonical mutations (\textit{katG} 315AGC-ACC and \textit{inha} promoter-15C-T) identified 89.5\% of resistance phenotypes in our collection. Inclusion of the 23 novel mutations reported here, and the previously documented point mutation in \textit{fabG1}, increased the sensitivity of these mutations as markers of INH resistance to 98\%. Only six (2\%) of the 332 resistant isolates in our collection did not harbor one or more of these mutations. The third most prevalent substitution, at \textit{inha} promoter position -8, present in 39 resistant isolates, was of no diagnostic significance since it always co-occurred with \textit{katG} 315. 79\% of our isolates harboring novel mutations belong to genetic group 1 indicating a higher tendency for this group to go down an uncommon evolutionary path and evade molecular diagnostics. The results of this study contribute to our understanding of the mechanisms of INH resistance in \textit{Mtb} isolates that lack the canonical mutations and could improve the sensitivity of next generation molecular diagnostics.

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\section*{INTRODUCTION}

\textit{Mycobacterium tuberculosis} (\textit{Mtb}) is the causative agent of tuberculosis (TB) and was responsible for the death of 1.5 million people in 2013.\textsuperscript{1} Multi-drug resistant TB (MDR-TB) strains are those that are resistant to at least rifampicin (RIF) and isoniazid (INH)—the two most effective first line drugs used to treat TB.\textsuperscript{1} Globally, 3.5\% of new and 20.5\% of previously treated cases (or a total of 300 000 cases) were estimated to be MDR-TB in 2013.\textsuperscript{1} Increasing resistance levels and rates of treatment failures in certain parts of the world are of major concern.\textsuperscript{1} Moreover, the availability of reliable and timely methods for the detection of drug-resistant TB strains has been inadequate as growth-based methods such as phenotypic drug susceptibility testing (DST) take weeks to months to complete and require laboratory infrastructure that is not widely available in countries with a high burden of TB.\textsuperscript{1,2} The World Health Organization (WHO) estimates that only about 45\% of all MDR-TB cases were diagnosed and notified in 2013.\textsuperscript{1} In part due to new rapid diagnostic methods, this rate has been continuously increasing from nearly 17\% in 2009.\textsuperscript{1}

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Clinical significance of INH resistance is most commonly due to missense mutations within the katG gene. Frequently, mutated S315T katG produces a functional catalase-peroxidase (katG) with enzymatic activity while highly deficient in its ability to form an INH-nicotinamide adenine dinucleotide adduct, reducing the INH toxicity and having little fitness cost to the bacterium.7–9 Alternatively, low-level resistance can emerge due to point mutations in the regulatory region of the inhA operon.10 For example, -15C-T mutation within the inhA promoter region, has been observed to increase inhA mRNA level to 20 times that of the wild type, resulting in inhA overexpression and an eight-fold increase in resistance to INH.11 While two polymorphisms, katG (S315T) and inhA (-15C-T), explain the majority of INH resistance in clinical isolates,10,12 other mutations carry the explanation for a subset of INH R cases that lack the two most common canonical mutations.13,14

Alternative mechanisms of INH R are not confined to katG and inhA. For instance, a synonymous mutation fabG1 L203L (CTG-CTA) (or fabG1 G609G) was first associated with INH resistance by Ramaswamy et al. in 2003.15 In 2014, Ando et al. described the role of this mutation as one that converts a segment of fabG1 into an alternative promoter for inhA and hence increases its expression level, suggesting these mutations may be important for next generation molecular diagnostics.16

In this study, we considered 366 clinical isolates collected in a joint effort through the Global Consortium for Drug-resistant TB Diagnostics (GCDD)17 from four high burden TB countries: India, Moldova, Philippines, and South Africa. We have identified 23 novel katG mutations associated with INH resistance and used mutagenesis to confirm the causal role of those that appeared in strains without a canonical mutation. Our results also indicate that genetic group 1 strains are more likely to traverse down an uncommon evolutionary path and thereby harbor uncommon mutations and hence more likely to evade molecular diagnostics. We believe the results of this research leads to a greater understanding of the full spectrum of mutations that can cause INH resistance, especially in those isolates that lack the canonical mutations, and therefore, to a more sensitive genotypic diagnostic of INH resistance.

MATERIALS AND METHODS

Sample set
Two sets of clinical isolates were used in this study. The first is referred to as the “archive set”. It contains 346 (316 INH R and 30 INH S) clinical isolates collected and sequenced as part of GCDD from India, Moldova, Philippines, and South Africa. Each isolate was collected from a different patient and therefore the set represents 346 independent isolates from the four countries. A second set, referred to as the “supplemental set” contained 20 additional clinical isolates (16 INH R and 4 INH S). These isolates were specifically selected because they harbored no canonical mutations in katG or the inhA promoter. In this article, we define canonical mutations as those occurring in codon 315 of katG or in positions -17, -15, or -8 of inhA promoter region. In total, this study considered 366 (332 INH R and 34 INH S) clinical isolates.

Archive sample collection strategy. The goal of GCDD was to obtain a large collection of M/extensively drug-resistant (XDR)-TB isolates that maximized diversity of M/XDR-TB phenotypes (i.e. DST profiles). Based on the global prevalence of M/XDR-TB and availability of large repositories of M/XDR-TB isolates, we focused our collection on India, Moldova, Philippines, and South Africa. For this study, we sequenced 316 INH R and 30 INH S of these isolates. INH resistance was determined using DST as described below. Details of sample selection and phenotypic characterization of these isolates can be found in Rodwell et al. (2014).12

Supplemental sample collection strategy. The goal of the collection of the supplemental isolates was to identify and study the mechanism of resistance in INH R clinical isolates that lacked any canonical mutations in katG or inhA promoter. INH resistance was determined using DST as described below. The minimum inhibitory concentration (MIC) of each isolate was also estimated as described below. In this set, 16 INH R and 4 INH S clinical isolates without any canonical mutations in katG or inhA were included (all from South Africa).

Sample representativeness. Samples from the archive set were drawn from M/XDR-TB repositories at (i) Hinduja National Hospital (PDHNH) in Mumbai, India representing Mumbai strains; (ii) Phthisiopneumology Institute (PPI) in Chisinau, the central unit of the National TB Control in Moldova, representing national TB strains in Moldova; (iii) Philippine Tropical Disease Foundation (TDF), representing Manila strains; and (iv) The National Health Laboratory Service of South Africa (NHLS), representing a national archive of South African TB strains.

Samples from the supplemental set were drawn from the NHLS similar to the archive set with a selection bias for isolates that had no canonical mutations in katG or inhA promoter region.

Phenotyping

Drug susceptibility testing of archive samples. Upon receiving isolates at University of California, San Diego (UCSD), each isolate was subjected to DST for INH on the Mycobacterial Growth Indicator Tube (MGIT) 960 platform, analyzed by EpiCenter software (BD Diagnostic Systems, Franklin Lakes, NJ, USA), using standard manufacturer protocol and WHO critical concentration (CC) of 0.1 mg/L for INH.18 Using MGIT 960, the DST of the isolates was also determined for six additional first- and second-line drugs (Supplementary Table S1).

Determination of MIC of supplement samples. MICs were determined using MGIT 960 system, according to the agar proportion method, looking for 99% inhibition of growth. Cultures were exposed to INH concentrations of 0.05, 0.1, 0.5, 3, and 10 mg/L. Therefore, an MIC level of 10, indicates a strain with an MIC level of 10 mg/L or greater. Values reported here as “≤10” indicate that there was not complete inhibition of growth (99%) at 10 mg/L of INH, but that the strain was struggling to grow at that concentration despite reaching the required growth units. Isolates reaching 100 growth units within a week of the control were marked as resistant at the concentration. Isolates not reaching 100 growth units were labeled as sensitive at that concentration.

Lineage determination

Lineage typing was performed using spoligotyping and MIRU as described in Garfein et al. (2015).19 In summary, using standardized methods,20–22 spoligotyping and 12 mycobacterial interspersed repetitive unit (MIRU-12) assay was determined. TB Insight’s TB-Lineage,23 and online lineage prediction tool was used for the analysis of the resulting Spoligotyping and MIRU-12 results and for lineage prediction using a knowledge-based Bayesian network.24
Sequencing
Whole-genome sequencing (WGS) was used as the primary sequencing technology for this project. The \textit{katG} gene (2223 bp) and \textit{inhA} promoter region (289 bp) were first examined for presence or absence of canonical mutations (\textit{katG} 315, \textit{inhA} -8, -15 and -17). Since the absence (or presence) of canonical mutations in isolates that harbor novel mutations were critical for this study, we have confirmed the WGS discovery (or lack thereof) of these mutations using the previously published Sanger sequencing\cite{12} and Pyrosequencing\cite{25} results from the same isolates. The WGS data (which cover the entire genes rather than small regions around canonical mutations) are being published here in this article for the first time.

Whole-genome sequencing. Pacific Biosciences single molecule, real-time sequencer (PacBio RS) was used for WGS. Since the \textit{Mtb} genome has a high GC content, this technology was chosen because it does not suffer from GC bias.

Sample preparation and DNA extraction. Clinical isolates were cultured on Lowenstein Jensen media, killed through exposure to ethanol and heat, lysed, and then DNA extracted.\cite{26} The supplemental sample set were grown on 7H10 agar, heat killed and DNA was isolated using the QIAGEN Genomic tip 100/g, cat. nr. 10243.\cite{27}

Library preparation. DNA libraries for PacBio (Pacific Biosciences, Melon Park, CA, USA) were prepared using PacBio’s DNA Template Prep Kit with no follow-up PCR amplification. Briefly, sheared DNA was end repaired, and hairpin adapters were ligated using T4 DNA ligase. Incompletely formed SMRTbell templates were degraded with a combination of exonuclease III and exonuclease VII. The resulting DNA templates were purified using SPRI magnetic beads (AMPure, Agencourt Bioscience, Beverly, MA, USA) and annealed to a two-fold molar excess of a sequencing primer that specifically bound to the single-stranded loop region of the hairpin adapters. The final sample resulted in library insert sizes of 2 kb in length. SMRTbell templates were subjected to standard SMRT sequencing using an engineered phi29 DNA polymerase on the PacBio RS system according to manufacturer’s protocol.

Post-sequencing analysis. Raw H5 files produced by the RS were processed by an in-house WGS pipeline developed for the Pacific Biosciences RS system. The Pac-DAP (Pacific Biosciences Data Analysis Protocol) pipeline was developed by the Bioinformatics and Medical Informatics Research Center (BIMIRC) of San Diego State University (SDSU). It employs a combination of SMRT Portal (version 1.3.3) (PacBio sequencer’s native software), GATK (version 2.2.2),\cite{28-30} SAMtools (version 0.1.18),\cite{31} BamTools (version 2.2.3),\cite{32} VarScan (Version 2.3.6),\cite{33,34} and in-house developed code for variant calling and housekeeping. In brief, the raw H5 data files of RS were imported into SMRT Portal. Reads from the H5 files were aligned to \textit{M. tuberculosis} H37Rv genome from NCBI by BLASR, SMRT Portal’s Basic Local Alignment. Quality scores were recalibrated and converted from Pacific Biosciences’ scoring metric into Phred quality scores by using GATK. SAMtools converted recalibrated BAM files into pileup files for VarScan’s variant caller and consensus generator. For the purpose of this study, only single nucleotide polymorphisms (SNPs) were considered and insertions and deletions were ignored as no significant number of insertions or deletions have been previously associated with INH resistance.

Sanger sequencing. Applied Biosystems 3730 XL Analyzer with BigDye Terminator v3.1 Cycle Sequencing Kit was used to sequence 12.3% of the \textit{katG} gene (274 bases) around codon 315.\cite{12,22} The complete \textit{inhA} promoter locus was sequenced, roughly 289 bases.\cite{13} Forward primers were used for sense strand sequencing and chromatograms were scored by the ABI base caller with the sensitivity set at Q20 (Supplementary Table S2). Any bases scored as “N” were visually read and a base letter was assigned. If the chromatogram was unclear, isolates were excluded. The relevant PCR products for sequencing were generated using the primer pairs as indicated in Supplementary Table S2.

Pyrosequencing. PyroMark Q96 ID system (Qiagen, Valencia, CA, USA) was used to perform Pyrosequencing of Sanger sequencing gene targets identified in \textit{katG} and \textit{inhA} promoter. Variant calling in Pyrosequencing was performed automatically with Identifier Software (Qiagen, Valencia, CA, USA) according to manufacturer specifications and procedures as described by Lin \textit{et al.} (2014).\cite{35} Two narrow ranges around \textit{katG} 315 (codons 312 through 316) and \textit{inhA} -15 (-4 through -20) positions were sequenced as described in Lin \textit{et al.} (2014).\cite{35} Pyrosequencing was performed at the California Department of Public Health, Microbial Diseases Laboratory.

SNPs discordance resolution. Two regions, 13 bases in \textit{katG} (codons 312-316) (2155164-2155176) and 17 bases in \textit{inhA} promoter (-4 to -20), were sequenced by all three technologies.\cite{35} Base calling in these regions required the additional condition of at least two of the three sequencing methods having to agree on the call. In this way, variant calling for canonical mutations (\textit{katG} 315, \textit{inhA} -8, -15 and -17) required the concordance of at least two of the three platforms.

Functional confirmation of the role of novel mutations in INH resistance
In this study, we have used \textit{Mycobacterium smegmatis} (due to its faster growth rate) to confirm the role of standalone novel \textit{katG} mutations in INH resistance. \textit{M. smegmatis} has been previously used as a surrogate for \textit{Mtb} in a variety of functional confirmation studies.\cite{36-44} Xu \textit{et al.} (2011) used \textit{M. smegmatis} to confirm the role of mutations in INH and ethionamide resistance.\cite{38} Parish \textit{et al.} (2007) demonstrated that \textit{M. smegmatis} can be used to assess the functional complementation of \textit{fabG1}.\cite{41} The same study determined that while \textit{M. smegmatis} is a good surrogate for \textit{Mtb}, \textit{Escherichia coli} is not.\cite{41}

\textit{M. smegmatis} \textit{katG} deletion mutant construction. The \textit{katG} gene from \textit{M. smegmatis} strain mc\textsuperscript{2}155, encoded by MSMEG\textsubscript{6384} was deleted from its genome by using allelic exchange as previously described.\cite{45} Fragments F1 (flanking the 5’ end of \textit{katG}) and F2 (flanking the 3’ end of \textit{katG}) were generated using PCR, using primer pair KGF1 (5’-GCC AAG CTC GAC CAG TTG C-3’) and KGR1 (5’-GGT ACT CGG TGC GGA ACT GC-3’), and pair KGF2 (5’-CAA TTC ACC ACT CCC GAA AGC ACA CAA CGA CCT GGA C-3’) and KGR2 (5’-GGT ACT CGG TGC GGA ACT GC-3’) respectively. F1 and F2 were linked together using F1 and F2 PCR products as templates in a PCR with primer pair KGF1 and KGR2, using the region of homology between primers KGR1 and KGF2 to generate a single product. PCR conditions were: initial denaturation at 98°C for 30 s, followed by 35 cycles consisting of denaturation at 98°C for 10 s, hybridization at 60°C for 15 s, and elongation at 72°C for 3 min. Phusion High-fidelity Polymerase\cite{46} was used for all reactions. The resulting product, F\textsubscript{R}2, was cloned into the pJET1.2 vector and its identity confirmed with

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Sanger sequencing. F₁R₂ was sub-cloned into the allelic exchange vector p2NIL to create plasmid pNKF₁R₂, where-after the PacI fragment from plasmid pGOAL17 was cloned into the PacI site of pNKF₁R₂ to create suicide construct pNKF₁R₂G. This plasmid was electroporated into M. smegmatis mc²155 and mutants selected in a two-step mutagenesis process by single cross-over mutant selection on Luria agar containing hygromycin (50 μg/mL), followed by selection of katG deletion mutants on sucrose-containing agar. Deletion of katG was confirmed with PCR and Southern hybridization, the latter employing labeled fragment F₁ as probe and detection with the aid of the ECL Direct Nucleic Acid Labelling and Detection System (Amersham).

**Complementation of M. smegmatis katG mutant.** Primer pair KGPromF (5′-TGG CGA GGC ACC CTG TCT GAC G-3′) and KGR (5′-GGT GCT GCG GCG GGT TGT GG-3′) and Phusion Polymerase were used to amplify a DNA fragment from M. tuberculosis clinical strain R35. This strain contains the wild-type katG gene with the phylogenetic marker mutation at codon 463. A fragment with this marker was selected specifically because the majority of novel mutations described here contains this marker. PCR was performed using an initial denaturation step at 98°C for 30 s, followed by 35 cycles consisting of denaturation at 98°C for 10 s, hybridization at 60°C for 15 s, and elongation at 72°C for 2.5 min. The product contained the wild-type M. tuberculosis katG gene, encoded by Rv1908c, as well as the promoter directly upstream of the katG gene and was cloned into pJET1.2. Sequence homology was verified with Sanger sequencing before the product was sub-cloned into plasmid mycobacterial integration vector pMV306, to generate integration construct pMV1908c. This construct was used to generate plasmids containing novel katG variants, using the components of the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA) and the modified site-directed mutagenesis method as described previously.⁴⁷ The presence of the mutations was confirmed using Sanger sequencing with primers promKF, RTB59, RTB83, and KGR, enabling sequence verification of the full length gene as well as the promoter. Primers to generate the various mutations are described in Supplementary Table S3. A variant containing the canonical S315T mutation, named pMV-S315T, was also constructed, and used as a resistance control for the phenotypic assay. The various plasmids were transformed into the katG deletion mutant and selected on Luria agar containing hygromycin (50 μg/mL). Plasmids were named pMV-X, with X denoting the number of the mutated codon from M. tuberculosis katG.

**Phenotypic characterization of INH resistance in M. smegmatis.** M. smegmatis katG wild-type mc²155, katG deletion mutant KGDAB, as well as complemented strains were grown overnight in Middlebrook 7H9-OADC (0.2% glycerol, Middlebrook oleic acid-albumin-dextrose-catalase (OADC), and 0.05% Tween 80), to reach an optical density (determined at 600 nm) of 0.8–1. The optical density was standardized to 0.1, where-after serial dilutions of each strain were made in Middlebrook 7H9 broth. Aliquots, 5 μL each, were spotted onto 7H10-OADC agar containing 12 μg/mL of INH. Plates were incubated for two to three days before colonies were visible. The presence of growth was regarded as an indication of resistance, while the absence or very faint growth indicated sensitivity toward INH. The mutant transformed with pMV1908c was used as a control for restoration of sensitivity, while the mutant transformed with pMV-S315T was used as a control for resistance to INH (Supplementary Figures S1–S4).

**RESULTS**

**Drug susceptibility testing**

Drug susceptibility testing of the archive isolates identified 30 INH⁸ and 316 INH⁹ isolates. Seven of the 316 INH⁸ isolates (2.2%) were mono-INH resistant and 301 were MDR-TB, of which 242 were XDR. Details of the resistance phenotypes are available in Supplementary Table S1. As mentioned before, the supplement set contained 16 INH⁸ and four INH⁹ isolates. In total, this study considered 332 INH⁸ and 34 INH⁹ isolates for WGS analysis. With our WGS approach, we were also able to identify polymorphisms in codon 463 allowing categorization of our clinical isolates into distinct lineage and epidemiological genetic groups. Breakdown of the observation of this mutation per country is shown in Supplementary Table S4. 191 of the 332 (or 58%) resistant isolates and 22 of the 34 (or 63%) susceptible isolates harbored a R463L mutation.

**Canonical mutations**

Two hundred and ninety-eight (89.5%) INH⁸ and two (5.9%) INH⁹ isolates contained one or both of the two (katG 315AGC-ACC and inhA promoter -15C-T) canonical mutations conferring INH resistance. The most frequent mutation found was S315T, which was present in 273 (82%) of resistant strains. Also two susceptible (by DST) strains harbored a canonical mutation (one had katG 315AGC-ACC and harbored inhA promoter -15C-T) (see the “DISCUSSION” section for more information about this discordance). Table 1 shows the breakdown of the 366 isolates included in this study. The numbers reported in Table 1 for all canonical mutations have been confirmed by at least two of the three sequencing platforms. Supplementary Figure S5 shows the prevalence of various Mtb lineages among our resistant isolates that harbored a canonical mutation.

**Other previously characterized katG mutations**

Seven resistant (and no susceptible) isolates harbored other previously characterized, non-canonical, resistance-causing katG mutations. Four of the seven also harbored a canonical mutation while three did not and hence the non-canonical mutations observed in the three isolates were the only explanation for resistance in katG or inhA.

**Novel mutations**

The remaining “unexplained” INH⁸ isolates (32 isolates) were of particular interest because of their lack of canonical and other previously characterized INH resistance causing mutations in katG and inhA promoter region. Within this subset of unexplained INH⁸ isolates, 14 novel katG substitutions were identified in 15 INH⁸ isolates (Table 2). It is important to note that a mutation in codon 64 was previously reported by Chan et al. in 2007.⁴⁸ We believe that this mutation was reported in the reverse direction and therefore not a true representation of codon 64 and the polymorphism we observe within our dataset.

Eleven of the 15 isolates harboring the listed novel katG mutations also harbored R463L (CGG-CTG) (Arg463Leu) polymorphism. The position of eight observed polymorphisms in katG codons 131,142, 162, 269, 306, 387, 394, 541 have been previously reported in INH⁸ isolates,⁷⁻⁵² however the specific polymorphisms we report at the eight loci are novel. These are: P131T(CCG-AGC), D142G(GAC-GGC), A162V(GCG- GTG), G269D(GGT-GAT), T306P(ACC-CCC), D387G(GAT-GGT), T394M(AGC-ATG), A541D(GCG-GAC). Furthermore, our group reported the discovery of T306P by Sanger sequencing in a recent manuscript.¹² Here we report that a broader examination of the rest of the katG gene and the inhA promoter region
using WGS of the isolate revealed no other resistance associated mutations in inhA or katG genes. We, therefore, describe and confirm (through mutagenesis) here the novel role of this mutation as a new mechanism of resistance.

In addition to the 14 novel katG mutations, nine additional novel katG amino acid substitutions were observed either in combination with katG S315T or with -15C-T inhA promoter mutation (Table 3). Two of these substitutions (N596S and Y597H) occurred together in a single isolate harboring D194G and the Y597H–N596S combination. Studies have shown that clinical isolates that harbor both the -15C-T inhA promoter and a katG mutation have higher MICs than either a single S315T katG or inhA promoter mutation. Interestingly, the majority of the novel mutations listed in Table 3 co-occur with inhA -15 substitution. Only two isolates, harboring D194G and the Y597H–N596S combination, of the nine mutations listed in Table 3 co-occur with a katG 315 substitution.

In total, 23 unique katG amino acid mutations were observed, 14 novel standalone katG mutations, an additional 9 novel katG mutations either in combination with S315T or -15C-T inhA promoter mutations. Lastly, seven previously characterized polymorphisms, 1335V, G299S, T275A, A264T, E127P, A110V, and A93V were also observed within the collected clinical isolates (Supplementary Table S5). Figure 1 displays the spatial proximity of the 23 mutations (Tables 2 and 3) to known resistance conferring mutations. A subset of novel standalone katG polymorphisms fell in close proximity to previously described katG substitutions suggesting a similar role in INH resistance (Figure 1). INH MICs were determined for the 16 resistant isolates from the supplemental set of isolates (Supplementary Table S6). These values are also included (in log scale) in Figure 1 for a visual correlation between the location of the mutations and their associated MIC levels.

### Table 1: Isolate stratification based on substitutions in katG and inhA promoter

| Observed mutations                                      | Number of INH<sup>a</sup> isolates | Number of INH<sup>b</sup> isolates |
|---------------------------------------------------------|------------------------------------|------------------------------------|
| katG S315T (AGC-ACC)                                   | 191                                | 1                                  |
| katG S315T (AGC-ACC) + inhA -15C-T                     | 28                                 | 0                                  |
| inhA -15C-T                                            | 24                                 | 1                                  |
| katG S315T (AGC-ACC) + inhA -8 (T-C or T-A)<sup>a</sup> | 39                                 | 0                                  |
| inhA -17G-T                                            | 1                                  | 0                                  |
| katG S315T (AGC-ACC) + inhA -17G-T                     | 8                                  | 0                                  |
| katG S315T (AGC-ACC) + inhA -34 (C-G or C-T)<sup>a</sup> | 2                                  | 0                                  |

Other previously characterized resistance conferring katG mutations and canonical mutation(s) occurred together in a single isolate S315T or with -15C-T amino acid substitutions were observed either in combination with katG or in positions -17, -15, or -8 of inhA promoter region.

### Table 2: Novel katG substitutions in isolates lacking canonical mutations

| Amino acid substitution(s) discovered in the katG | MIC µg/mL | Phenotypic grouping<sup>a</sup> | NO of INH<sup>b</sup> isolates |
|--------------------------------------------------|-----------|---------------------------------|--------------------------------|
| Y64S (TAT-TCT)<sup>c</sup>                       | 3         | INH<sup>a</sup> other DST unknown | 1                              |
| Y95C (TAC-TGG)<sup>c</sup>                       | 0.5       | INH<sup>a</sup> other DST unknown | 1c                             |
| P131T (CCG-ACG)<sup>c</sup>, b                    | 0.5       | INH<sup>a</sup> other DST unknown | 1c, a                          |
| A139P (GCC-CCC)<sup>c</sup>                       | >10       | INH<sup>a</sup> other DST unknown | 1c                             |
| D142G (GAC-GGC)<sup>c</sup>, b                    | >10       | INH<sup>a</sup> other DST unknown | 1c                             |
| A162V (GCG-GTG)<sup>c</sup>, b                    | 3         | INH<sup>a</sup> other DST unknown | 1c                             |
| G269D (GGT-GAT)<sup>c</sup>, b                    | >10       | INH<sup>a</sup> other DST unknown | 1c                             |
| T306P (ACC-CCC)<sup>c</sup>, b                    | Not done  | MDR<sup>c</sup>                  | 1c                             |
| R385W (CGG-TGG)<sup>c</sup>                       | >10       | INH<sup>a</sup> other DST unknown | 1c                             |
| D387G (GAT-GGT)<sup>c</sup>, k                    | ≤10       | INH<sup>a</sup> other DST unknown | 2c                             |
| T394M (ACG-AGT)<sup>c</sup>, f                    | Not done  | XDR                             | 1                              |
| Q439P (CAG-CCG)<sup>c</sup>                       | Not done  | MDR                             | 1                              |
| F483L (TTC-CTC)<sup>c</sup>                       | 3         | INH<sup>a</sup> other DST unknown | 1c                             |
| A541D (GCC-GAC)<sup>c</sup>, b                    | >10       | INH<sup>a</sup> other DST unknown | 1c                             |

<sup>a</sup> Isolates that are INH<sup>a</sup> and Rif<sup>b</sup> susceptible to the remaining five drugs are labeled as MDR. MDR<sup>c</sup> are pre-XDR isolates that are INH<sup>a</sup>, Rif<sup>b</sup>, and resistant to at least a fluoroquinolone (FQ) or an aminoglycoside but not to both.

<sup>b</sup> Denotes polymorphisms whose role in causing resistance has been confirmed through mutagenesis.

<sup>c</sup> Denotes isolates that harbor katG R463L lineage marker.

<sup>d</sup> Denotes novel polymorphisms at previously reported positions. **

<sup>e</sup> Denotes isolates that also harbor fabG<sup>esCOD</sup>.

<sup>f</sup> Denotes polymorphisms whose mutagenesis experiments were not successful. These are A139P and T394M.

<sup>g</sup> Denotes polymorphisms whose role in causing resistance has been disproved through mutagenesis. These are R385W and D387G.
Mutations in fabG1

The role of the recently described alternative mechanism of INH resistance through fabG1g609a was also investigated among our isolates. Supplementary Table S7 shows the grouping of the isolates that harbored this mutation. Fifteen resistant and no INH susceptible isolates harbored this mutation. Of the 15, 10 harbored a canonical katG315 mutation but five did not. Of the five, two harbored a novel mutation in katG reported above, namely, P131T and T306P, and one harbored a previously described but rare mutation in katG, namely, G299S. Mutagenesis experiments would be able to elucidate whether the observed resistance in these isolates is solely due to the fabG1 mutation, the katG mutations, or both having an epistatic effect to cause a higher resistance level. Two remaining isolates did not harbor any mutations in katG or inhA promoter other than the fabG1 and the katG463 lineage marker. INH resistance in the remaining two isolates is probably due to fabG1g609a, as no other known INH resistance conferring markers were detected by the three sequencing methods in these previously unexplained resistant cases. Both of these isolates harbor katG463 lineage marker and are from South Africa.

Mutations not conferring resistance

For the completeness of this manuscript, we also report katG mutations observed in INH8 isolates, and synonymous katG substitutions. Supplementary Table S8 lists two substitutions in katG gene of INHS isolates. Neither of these substitutions have been previously reported, although a P280H substitution (same position but different substitution as our observed P280S) was reported by Gagneux et al. (2006) as conferring INH resistance.7 Our isolate is mono-RIFR, and therefore we believe our substitution does not confer resistance. Supplementary Table S9 lists the synonymous mutations observed in INHR isolates. Since these synonymous SNPs do not cause any change in the structure of the protein, it is unlikely they confer resistance.

Isolates with no katG, inhA, or fabG1 resistance conferring mutations

As Table 1 indicates, eight (2%) INH8 and 32 (94%) INHS isolates did not harbor any mutations in the three genes studied in this project except for the lineage marker in katG codon 463. The eight resistant
is isolates are of great interest for discovery of alternative mechanism(s) of resistance (see the “DISCUSSION” section).

Confirmation of causal role in resistance
To verify if the identified novel standalone mutations confer resistance to INH in mycobacteria, we completed a series of mutagenesis experiments in M. smegmatis where we deleted katG, encoded by MSMEG_6384 and showed that the absence of this gene caused it to grow at a higher concentration of INH (Supplementary Figure S1). Complementation of the mutant with the wild-type katG gene from M. tuberculosis restored sensitivity to INH, whereas complementation with a variant encoding S315T did not restore sensitivity to the mutant (Supplementary Figure S2). Similarly, complementation with novel M. tuberculosis katG variants Y95C, P131T, D142G, A162V, T306P, Q439P, F483L, and A541D did not restore sensitivity to INH (Supplementary Figure S3), indicating these mutations confer resistance. A previously described variant, A264T, also did not restore sensitivity to INH, confirming its role in INH resistance.58 In contrast, complementation with a wild-type katG (which contained a polymorphism at 463), or the variants with mutations in codons R385W (Supplementary Figure S3) or D387G (Supplementary Figure S4) restored sensitivity to INH indicating that they do not appear to play a role in INH resistance.

DISCUSSION
From our collection of 332 INHR and 34 INHS clinical isolates, 89.5% of INH resistance could be explained by the two mutations of katG S315T, inhA promoter -15C-T mutations, or a combination of these two mutations. One isolate determined to be susceptible to INH by phenotypic DST harbored the katG S315T mutation and another harbored the inhA promoter -15C-T. The presence of both mutations was confirmed by all three sequencing platforms. We believe that the genotypic-phenotypic discordance may be due to variation in growth around the cut point of DST, although heterogeneity could be another cause of the discordance.

It is interesting to note that the third most prevalent mutation (present in 39 of 332 INHR isolates or 12%) at inhA promoter position -8 appeared to have little diagnostic significance in our isolates as they always co-occurred with katG315. The same is also true for mutations occurring at inhA promoter position -34. Because of the geographic variability of the prevalence of the canonical mutations conferring INH resistance, the sensitivity of molecular diagnostics for detecting INH resistant phenotypes can be variable.5 The discovery of 14 novel, standalone katG mutations could significantly improve the sensitivity of next generation molecular diagnostics that include these mutations, especially in regions where these mutations are more common (e.g., South Africa). In our dataset, using all of the mutations demonstrated to confer resistance, we were able to explain 326 of 332 INHR (98%) of INH resistant phenotypes by a katG, inhA promoter, or a fabG1mutations. This is a notable improvement over the commonly reported sensitivity of molecular diagnostic in the high eighties to low nineties.12,61,62

While all but two canonical mutations, katG S315T and inhA promoter -15C-T, seem not to be useful in diagnostics (since they co-occur with those mutations), we believe that they are important in prognosis of the disease. Previous work has shown that combination of inhA promoter and katG results in higher MIC levels.10,63 We believe the same effect may be taking place for combination of novel mutations reported and canonical mutations in inhA promoter or fabG1mutations. A higher resistance level of these combinations for these isolates would be indicative of possible new molecular interactions in katG and in INH resistance. Protein folding in combination with mutagenesis and enzymatic studies directly in Mtb would have to elucidate the specific molecular mechanisms and their effects on the resistance level.

Among isolates that harbor canonical mutations some interesting lineage patterns have emerged. These patterns are displayed in Supplementary Figure S5. First, the Indo-Oceanic group in our set displayed much greater tendency to only harbor an inhA promoter mutation as opposed to a katG mutation. 42% of Indo-Oceanic isolates belong to this group. The distant second was the Euro-American group, 5% of which harbored only an inhA promoter mutation. 68% of Euro-American group harbored a mutation in katG and one in inhA promoter. The distant second was the Beijing group in which 33% of the isolates harbored a mutation in both genes.

Codon 463 in katG has been shown not to be associated with resistance to INH and is commonly used to categorize clinical isolates into members of genetic groups 2 or 3, by a Arg 463 substitution, or genetic group 1, by a Leu 463 substitution.60 As mentioned before, 38% of all resistant isolates and 63% of all susceptible isolates from four countries in our study harbored a R463L mutation and therefore belong to genetic group 1. Importantly, 19 of the 24 (or 79%) isolates harboring a novel mutation also harbored R463L and hence belonged to genetic group 1. The higher percentage of R463L among resistant isolates harboring a novel mutation (79% as compared to 58% for all resistant isolates from four countries or 59% for all resistant isolates from South Africa which is where most of the isolates with novel mutations came from) may indicate a higher tendency for genetic group 1 to take an uncommon evolutionary path and feature one of the rare resistance conferring mutations. This hypothesis is further supported by the fact that 14 of the 15 (93%) isolates that harbor fabG1 also harbored R463L. This would indicate more frequent evasion from molecular detection by genetic group 1 and lower sensitivity of molecular tests in regions of the world where genetic group 1 is more prevalent.

With the recent discovery of multiple additional resistance conferring mutations in katG by multiple investigators,16,64–66 it is a concern that a more widespread use of existing molecular diagnostics that miss these mutations might impose an artificial selection process where the mutants with canonical mutations are detected and eradicated through appropriate therapy but those with these novel variants go undetected and continue to spread. This may ultimately drive the Mtb INH-resistant population evolution from the current “hotspot” model (most mutations in a few loci in close proximity to each other) to more of a “whole-gene” convergence model. The scatter pattern of the novel mutations reported in this article suggests that a “multi-hotspot” model may also be a possibility. Much more representative data are needed for a more certain determination of these trends and emphasizes the need for global genomic surveillance of resistant phenotypes and for more flexible diagnostic platforms with the ability to interrogate broad regions of the genome.

As mentioned before, the remaining eight INHR isolates (2% of all INHR isolates) did not have katG, inhA promoter, or fabG1 mutations. We therefore explored the remainder of the genome looking for mutations previously associated with INH resistance that could potentially explain their phenotype. A subset of the “unexplained” 2% of INHR isolates were found to possess either an inhA H481Q or a fabG1 mutations, both of which have been previously identified in INH-resistant Mtb isolates.67 or an accD D229G mutation shown to play a role in the building blocks for de novo fatty acid biosynthesis.
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by fatty acid synthase I (FAS I) that produce meromycolic acids. We believe the latter is worthy of further investigation as mycolic acids are essential for the survival, virulence, and antibiotic resistance of the Mtb. It is clear that further work is needed to investigate and validate the true cause(s) of resistance for the remaining 2% of our isolates not containing the mutations traditionally associated with INH resistance.

Finally, our results have confirmed that some mono-INH resistance isolates do lack the canonical mutations and instead harbor novel mutations reported in this article. Patients infected with Mtb isolates harboring these mutations would go undetected by the currently available molecular tests for INH resistance and would be prime candidates for conversion to MDR-TB.

M. smegmatis is a convenient proxy for understanding functional consequences of mutations in Mtb because of its higher growth rate and its noninfectious nature. M. smegmatis, however, is not an accurate model for estimation of functional levels (e.g., resistance levels) in Mtb. As such, it is possible that mutations that do not cause (or cause low levels of) resistance in M. smegmatis, cause detectable and consequential levels of resistance in Mtb. For this reason, mutagenesis directly in Mtb, or at least in closer models such as H37ra would provide a more accurate functional-level estimation. In particular, the noncausative role of the two mutations R385W and D387G in INH resistance, as determined by H37ra, suggests that mutations in these genes may not cause INH resistance in Mtb.

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