AAC Accepted Manuscript Posted Online 9 October 2017
Antimicrob. Agents Chemother. doi:10.1128/AAC.01461-17
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1 Whole transcriptome and genomic analysis of extensively drug-resistant *Mycobacterium*
2 *tuberculosis* clinical isolates identifies downregulation of *ethA* as a mechanism of
3 ethionamide resistance

4

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13

14 XDR-TB Transcriptomics and Ethionamide resistance

15

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19

20 Abstract: 220 words
21 Text (Excluding Figure Legends and References): 4165 words

22

23 **ABSTRACT**



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24 Genetic based drug susceptibility testing has improved the diagnosis of drug-resistant
25 tuberculosis, but is limited by our lack of knowledge of all resistance mechanisms. Next
26 generation sequencing has assisted in identifying the principal genetic mechanisms of
27 resistance for many drugs, but a significant proportion of phenotypic drug resistance is
28 unexplained genetically. Few studies have formally compared the transcriptome of
29 susceptible and resistant *M. tuberculosis*. We carried out comparative whole genome
30 transcriptomics on extensively drug-resistant (XDR) clinical isolates using RNA-
31 sequencing (RNAseq) to find novel transcriptional mediated mechanisms of resistance.
32 We identified a t-11c promoter mutation that reduces expression of a monooxygenase
33 (EthA) that activates ethionamide. Using a flow-cytometry based reporter assay, we show
34 that reduced transcription of *ethA* is not due to transcriptional repression by *ethR*.
35 Clinical strains harbouring this mutation were resistant to ethionamide. Other *ethA*
36 promoter mutations were identified in a global genomic survey of resistant *M.*
37 *tuberculosis* strains. These results demonstrate a new mechanism of ethionamide
38 resistance that can cause high-level resistance when combined with other ethionamide
39 resistance conferring mutations. Our study revealed many other genes which were highly
40 up or down regulated in XDR strains, including a toxin-antitoxin module (*mazF5 mazE5*)
41 and tRNAs (*leuX* and *thrU*). This suggests more global transcriptional modifications have
42 also occurred in XDR strains that could contribute to resistance or maintaining bacterial
43 fitness.

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45

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## 46 INTRODUCTION



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47 ***Mycobacterium*** *tuberculosis*, the causative agent for tuberculosis (TB), has progressively
48 developed resistance to the most effective first and second-line anti-tuberculosis drugs.(1)
49 Patients infected with extensively drug-resistant (XDR) strains (resistant to the
50 fluoroquinolones and aminoglycosides in addition to rifampicin and isoniazid that define
51 multi-drug resistance [MDR]) have extremely high mortality despite long and intensive
52 treatment regimens.(2, 3) Ultimate control of drug resistance will require multiple
53 interventions, one of which will be individualized therapy based on rapid comprehensive
54 drug susceptibility testing (DST).
55
56 **Current** molecular genetic based tests, such as the Gene®Xpert MTB/RIF and
57 GenoType® MTBDRplus, have accelerated the clinical detection of known mutations
58 causing rifampicin (RIF) and/or isoniazid resistance.(4, 5) These and other genetic tests
59 only detect MDR TB and a limited number of mutations associated with resistance to
60 second-line drugs.(6) Whole genome sequencing (WGS) has the potential to rapidly
61 detect all possible drug resistance conferring mutations.(7) However recent studies have
62 demonstrated that genotypic DST using WGS lacks sensitivity for the detection of many
63 second-line resistances including to fluoroquinolones.(8–11) Improving the sensitivity of
64 genetic susceptibility testing will only be possible with a more comprehensive
65 understanding of the genetic determinants of drug resistance.
66
67 **Our** current understanding of drug resistance in *M. tuberculosis* has developed through
68 studying resistance mutants isolated *in vitro* and the accumulation of mutations in
69 resistant clinical isolates.(12) These studies have identified various genetic mechanisms

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70 of resistance including target modification, loss of enzymatic function required to activate
71 prodrugs, and altered drug efflux.(13, 14)
72
73 **In** addition to intragenic mutations there is increasing evidence that alterations to gene
74 transcription are an important mechanism of conferring drug resistance. Promoter
75 mutations which result in upregulation of *inhA*, that encodes the target for isoniazid, were
76 the first to be described.(15) Pyrazinamide (PZA) resistance has been associated with
77 mutations in the regulatory region upstream of *pncA,* the enzyme responsible for
78 activating PZA.(16–18) Aminoglycoside cross-resistance in *M. tuberculosis* can arise due
79 to mutations in the regulatory region of *whib7* (encoding a transcriptional activator)
80 which results in increased expression of *eis* (which acetylates and inactivates kanamycin),
81 as well as *tap* (which encodes an efflux pump that extrudes streptomycin).(19) *eis*
82 promoter mutations have also been described. Recently, cross-resistance between
83 clofazamine (CFZ) and bedaqualine (BDQ) was shown to be due to mutations within
84 *Rv0678* (20, 21), a transcriptional repressor, which results in derepression and
85 upregulation of the multi-substrate efflux pump *mmpL5*.
86
87 **Despite** the discovery of these varied transcriptionally driven mechanisms of resistance,
88 there have been few systematic whole genome transcriptional comparisons of suitably
89 matched susceptible and resistant *M. tuberculosis* strains, and none to date using RNA-
90 sequencing (RNAseq). In this study, we therefore selected phylogenetically closely
91 related susceptible and resistant clinical strains and subjected them to comparative
92 transcriptomics using RNAseq to identify novel mechanisms of resistance.
93



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94 **RESULTS**

95 **Comparative Transcriptomics**

96 **In** order to identify novel mechanisms of resistance mediated at the level of transcription,
97 we subjected drug resistant and drug susceptible strains of *M. tuberculosis* to comparative
98 transcriptomics using RNA sequencing (**Table 1**). We reasoned that strains with highly
99 complex resistance profiles were most likely to have acquired mutations resulting in
100 transcriptional changes. Using a whole genome based phylogenetic analysis, we
101 identified 3 XDR clinical isolates from a well-documented outbreak in KwaZulu-Natal
102 and a closely related drug susceptible strain to act as a control.(1) All strains were from
103 the LAM4 branch of Lineage 4. In pairwise comparisons, the 3 XDR strains varied by 7
104 or less SNPs from each other (**Figure 1A**). The maximum SNP difference between the
105 drug susceptible and a XDR strain was 76 SNPs, of which 6 occurred in known drug
106 resistance conferring genes.
107
108 To determine if there were global transcriptional differences between our strains, we first
109 carried out hierarchical clustering of their transcriptional profiles. This separated the
110 expression profiles of the three drug-resistant strains from the susceptible control **(Figure
111 1B**). To identify genes either up or down regulated in the XDR strains, we performed
112 pairwise comparisons for each resistant strain with the drug susceptible control. In the
113 resistant strains relative to the susceptible control, up to 40 genes were significantly over
114 or under expressed at the 95% confidence level ($p\text{-value} \leq 0.05$), and up to 10 genes at
115 the 99% level ($p\text{-value} \leq 0.01$) **(Table S1).** Importantly, in all three pairwise
116 comparisons, *inhA* showed a greater than 8-fold up-regulation in gene expression in the
117 resistant strains at the 99% confidence interval. All three resistant strains harboured a t-8a

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118 mutation in the promoter region of *fabG1,* which is known to cause up-regulation of *inhA*.
119 The detection of this transcriptional change therefore acted as an internal validation of
120 our approach. Apart from *inhA* there were no other genes that were significantly
121 upregulated in all three comparisons. There were two genes, *fabG1* (also in the *inhA*
122 operon) and Rv1761c (a gene of unknown function), that had expression levels
123 significantly different in two strains relative to the susceptible control.
124
125 **After** defining differential gene expression at the statistically significant levels (95% and
126 99% confidence intervals), we extended our analysis to all genes that had a high mean
127 fold change (≥ 7 fold up/down) in transcripts relative to the susceptible control (**Figure**
128 **1C and Table S2**). In addition to *fabG1* and *inhA*, we found that 5 other genes fell into
129 this classification: *mazF5*, *mazE5* encoding a toxin-antitoxin module; two tRNAs (*leuX*
130 *,thrU*) and *ethA*. *ethA* was of particular interest as it encodes a monooxygenase required
131 for the activation of the prodrug ethionamide (22, 23), a key component of MDR
132 treatment. Loss of function mutations in *ethA* result in ethionamide resistance.(23, 24)
133 *ethA* was significantly downregulated following Benjamini Hochberg correction in one of
134 our pairwise comparisons described above.
135
136 **Comparative genome-transcriptome analysis**
137 **In** order to understand the genetic basis of the transcriptional changes defined by our
138 RNAseq experiments we used comparative genomics to identify mutations located in
139 intergenic regions associated with genes that were highly over or under expressed in our
140 resistant strains relative to our susceptible control. This analysis identified an intergenic
141 mutation at position –11 (t to c) relative to the start codon of *ethA*. The detected mutation

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142 was located within the promoter region of *ethA* as well as within the binding domain of
143 the divergently expressed transcriptional regulator *ethR* that is known to repress *ethA* (25)
144 (**Figure 2).** The location of the mutation suggested it could lead to down-regulation of
145 EthA by: i) directly reducing *ethA* transcription independent of *ethR* regulation ii)
146 increasing *ethR* transcription leading to repression of *ethA*, or iii) affecting binding of
147 *ethR* leading to increased repression of *ethA* transcription.
148
149 **Functional characterisation of *ethA* and *ethR* promoters**
150 **To** functionally determine if the t–11c mutation influenced either *ethA* or *ethR*
151 transcription, we used a dual-colour fluorescent protein promoter assay. The episomal
152 construct pLDW-DC* has a constitutively expressed TagRFP and a promoterless
153 Emerald GFP protein in front of which promoters with or without mutations can be
154 cloned. Promoter activity is expressed as the ratio of green to red fluorescence
155 normalizing for any variability in plasmid number. To validate our approach, we used the
156 *fabG1-inhA* promoter with and without the g-17t mutant promoter sequence of *inhA*. The
157 construct harbouring the g-17t mutant promoter sequence of *inhA* resulted in a 3.4 fold
158 increase in the ratio of the mean fluorescent intensity (MFI) relative to wild-type (**Figure**
159 **3A**).
160
161 **We** then assayed constructs with the wild-type 250 bp upstream region of *ethA* or *ethR,*
162 and 2 matched mutant constructs with either the t–11c mutation (relative to *ethA*) or the
163 corresponding t– 65c in the *ethR* construct (**Figure 3B**). We observed no significant
164 change in the MFI ratio between the two *ethR* promoter constructs. In contrast the t-11c
165 mutant promoter resulted in an MFI ratio that was significantly lower than with the wild-



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166 type control. These results suggest that the t–11c intergenic mutation does not affect
167 transcription of *ethR*, but does diminish expression of *ethA* to levels that could result in
168 ethionamide resistance.
169

170 ***ethA* expression in clinical isolates of *M. tuberculosis***

171 **To** confirm the transcriptional changes identified by RNAseq in strains harbouring the t-
172 11c mutation, we used quantitative qRT-PCR to measure the expression levels of *ethA* in
173 clinical isolates **(Table S3)**. In the 5 strains tested with an *ethA* t-11c promoter mutation,
174 relative normalized expression levels of the monooxygenase were significantly lower or
175 close to zero compared to control strains. Strains tested with the *inhA* promoter
176 mutations all had increased relative normalised expression of *inhA* compared to those
177 without the mutation **(Figure 4).**
178

179 ***ethA* promoter mutations and ethionamide resistance in clinical isolates**

180 **To** determine if *ethA* promoter mutations were associated with clinical resistance, we
181 tested a panel of clinical isolates, which based on genome analyses harboured putative
182 ethionamide resistance conferring mutations, for quantitative ethionamide susceptibility
183 **(Table 2).** The panel included three strains with the t–11c intergenic mutation that had no
184 other mutations previously associated with clinical ethionamide resistance (*inhA*
185 promoter mutations and intragenic mutations in *ethA*, *ethR, ndh* and *mshA*).(26) Recently,
186 loss of function mutations in another *M. tuberculosis* monooxygenase *mymA* (Rv3083)
187 have been proposed as an additional resistance mechanism (27). Interestingly during our
188 selection of strains, we were able to identify a group of isolates with a deletion spanning
189 *mymA* (**Figure S1**). Sequence confirmation in five of these strains showed an identical



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190 deletion of 2891bp, indicating a unique event polymorphism suggestive of clonal
191 expansion. Strains with this mutation were included in our analysis.
192
193 **The** MIC for strains that only had the *ethA* t-11c promoter mutation ranged from 5 – 20
194 mg/L, showing a low-level resistance to ETH (**Table 2**). However, in combination with
195 the t-8a *inhA* promoter mutation, we observed higher levels of resistance suggesting the
196 phenotypic consequences of the two promoter mutations are additive. The two strains
197 with only *mymA* deletions had MICs of 2.5 and 5 mg/L, only marginally elevated relative
198 to the MICs of strains without any ethionamide drug resistance conferring mutations
199 (1.25 – 2.5 mg/L).
200

201 **Global Distribution of *ethA* promoter mutations**

202 **In** order to determine how widespread *ethA* promoter mutations are among clinical *M.*
203 *tuberculosis* isolates, we exploited a recent genome analysis of globally isolated drug-
204 resistant and susceptible strains (28). From a total of 5310 strains, we identified 402 with
205 a mutation relative to H37Rv within the *ethA-ethR* intergenic region (**Table S4**). One
206 hundred and thirty-nine of these were the t-11c mutation, all of which were identified in
207 South African derived lineage 4 isolates. The most common mutation was the a-7g found
208 in 212 strains, nearly all of which (205) were from Eastern Europe. Eleven other
209 infrequently occurring mutations were identified, but one of these also mapped to the -11
210 site (t-11g). Using parsimony to define independent mutational events across the
211 phylogeny (29), we found that the a-7g mutation had independently evolved at least 32
212 times, suggesting this mutation was under selective pressure and supporting a role in
213 conferring drug resistance. In contrast the t-11c was predicted to have evolved only once,



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214 which is compatible with ongoing transmission and clonal expansion of XDR strains
215 from South Africa in which the mutation was found.
216
217 **DISCUSSION**
218 **The** aim of our study was to use a comparative whole genomic-transcriptomic approach
219 to identify novel mechanisms of resistance mediated at the transcriptional level. We were
220 able to identify a promoter mutation upstream of *ethA* that we confirmed by our dual-
221 colour promoter assay and quantitative RT-PCR leads to reduced transcription of *ethA*,
222 which encodes a monooxygenase that activates the prodrug ethionamide.(22, 23) Strains
223 harbouring only this t-11c mutation and no other ethionamide resistant determining
224 genotypes were resistant to ethionamide (MICs ranging from 5 – 20mg/L) indicating this
225 mutation should be included in genetic based diagnostic tests. In support of this, a recent
226 genome wide association analysis also reported an association between the t-11c and
227 ethionamide resistance.(8)
228
229 **In** our analysis of the global distribution of *ethA*-*ethR* intergenic mutations, we identified
230 the t-11c mutation solely in South African strains. This data set however, consisted of
231 isolates from only 43 countries and notably lacked representation from several regions of
232 the world where TB is epidemic, such as South America. Nonetheless a previous study,
233 using direct sequencing of drug resistance loci, detected five different variants in the
234 promoter region of *ethA*, one of which was a t-11c mutation found in an isolate from Peru
235 (30). There was however no clinical data available to rule out whether the patient in
236 question had any travel history to South Africa. We can therefore not confirm whether
237 this mutation is geographically restricted. The a-7g mutation was more dispersed, but the

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238 majority of strains harbouring this mutation were from Eastern Europe. A previous study
239 reported the phenotype of 172 strains with the a-7g mutation and only 56 of these were
240 resistant to ethionamide.(31) This could be due to inconsistencies in drug susceptibility
241 testing, or the level of resistance conferred lying close to the break point used in
242 susceptibility testing but suggests that not all mutations within the intergenic *ethA*-*ethR*
243 result in similar levels of resistance that we observed with the t-11c mutation. (30)
244
245 **Few** studies have used quantitative drug susceptibility testing to correlate ethionamide
246 MIC with genotype (32) so it is unclear to what extent individual mutations contribute to
247 resistance and how they might interact. Although there may be additional mechanisms of
248 ethionamide resistance, yet to be identified, our results suggest that the t-11c mutation
249 causes a modest increase in ethionamide MIC but in combination with an *inhA* promoter
250 mutation (considered to cause low-level ethionamide resistance (24)) leads to high level
251 resistance. Among the other ethionamide resistant strains assayed, most had more than
252 one mutation potentially contributing to their increased MIC. The pathway to clinical
253 ethionamide resistance may therefore be the step-wise accumulation of multiple
254 mutations rather than the selection of a single high-level resistance conferring mutations
255 as seen with some other anti-tuberculosis drugs.
256
257 **In** the panel of clinical isolates we selected to evaluate the phenotype of the t-11c
258 mutation, we identified polymorphisms in other genes implicated in ethionamide
259 resistance. We found four mutations at three positions in the *inhA* promoter region all of
260 which have been previously described.(33) One of these strains had a t-8g *inhA* promoter
261 mutation in combination with a non-synonymous mutation (V18A) in *ndh* which encodes

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262 a type II NADH dehydrogenase. Mutations in *ndh* can result in increased levels of NADH
263 and reduce binding of the isoniazid and ethionamide NAD adducts to their target
264 InhA.(33) However this strain had a low MIC suggesting neither of these mutations
265 causes high-level resistance.
266
267 **We** cannot rule out the existence of other ethionamide resistance mechanisms in our
268 strains. EthA is one of 30 other monooxygenases within the *M. tuberculosis* genome (24)
269 and a recently characterized monooxygenase, *mymA* (Rv3083) (27) was proposed as an
270 additional enzyme responsible for the activation of ethionamide. We identified strains
271 with a 2891 bp deletion spanning *mymA*, *lipR* and half of *Rv3085* (**Figure S1**). Two of
272 these strains had no other known mutations associated with ethionamide resistance but
273 were susceptible to ethionamide employing a standard MIC cut-off (**Table 2**), suggesting
274 *mymA* is not important for drug resistance in clinical isolates.
275
276 **Our** initial comparative transcriptional analysis only identified a limited number of genes
277 whose expression was statistically different from the control. This may have been due to
278 increased variability associated with propagating clinical isolates in culture media. We,
279 therefore, looked at genes whose expression was highly divergent in the resistant strains
280 in all pair wise comparisons. In addition to *ethA* this identified *mazF5* and *mazE5*, which
281 encode a toxin antitoxin system, one of nine MazEF homologues in *M. tuberculosis*. A
282 triple null mutant of *mazF3*, *F6* and *F9* was less able to survive antituberculosis drugs
283 (34) so potentially these systems could be involved in mediating resistance, although it is
284 unclear how downregulation of *mazE5* would influence drug susceptibility. Two tRNAs,
285 *leuX* and *thrU*, were amongst the genes most highly upregulated in the resistant strains.



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286 Beyond a fundamental role in translation, tRNAs, and their degradation products have
287 been shown to regulate stress responses and adaptive changes in translation.(35) It is
288 therefore conceivable that the upregulation of these two tRNAs may be a manifestation of
289 more global regulatory changes that have occurred during the evolution of drug
290 resistance. Future studies comprising strains from different outbreaks and lineages are
291 however needed to determine whether these transcriptional changes are limited to the
292 XDR outbreak from KwaZulu-Natal in 2005 (1).
293
294 **The** treatment of MDR-TB is currently undergoing a revolution with the introduction of
295 new drugs and regimens.(36) WHO has recently approved the use of a 9-month short
296 course of therapy, and the 4-month intensive phase of this regimen includes ethionamide
297 (or its analogue prothionamide). Although the contribution of individual drugs to
298 treatment efficacy is unclear, it is recommended that short course treatment should be
299 withheld from MDR-TB patients with pre-existing resistance to any individual drug. Pre-
300 treatment screening for ethionamide resistance is therefore critical for the implementation
301 of short course MDR treatment. However phenotypic susceptibility testing for
302 ethionamide is notoriously difficult.(37) Our results contribute to the development of a
303 genetic based resistance test, but further studies are required to define the interaction of
304 diverse mutations and drug resistance conferring loci as well as establishing a clinical
305 relevant critical concentration for ethionamide.
306
307 **MATERIALS AND METHODS**
308 **Strains and growth conditions**

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309 **Three** XDR and 1 fully drug susceptible clinical isolate from the LAM4 (KZN)
310 spoligotype of *M. tuberculosis* (**Table 1)** were obtained from archived cultures that had
311 been previously genome sequenced from single colonies.(1) Cultures were grown in
312 triplicate at 37˚C in BD Difco$^{TM}$ Middlebrook 7H9 broth supplemented with BBL$^{TM}$
313 Middlebrook OADC enrichment media, 0.5% glycerol and 0.01% Tween 80, with
314 continuous shaking at 200rpm. Additional strains were selected from the same collection
315 based on specific *ethA*, *ethR*, *inhA*, and *mymA* genotypes (**Table 2**).(1)
316
317 **RNA extraction and quality control**
318 ***RNA Extraction.*** RNA was harvested from 25ml cultures grown to an $OD_{600}$ of between
319 0.5 – 0.8, using a modified TRIzol method.(38) Briefly the cultures were centrifuged at
320 4000 rpm for 20 minutes at 25°C and the pellet re-suspended in 1ml of TRIzol® reagent
321 (Invitrogen, USA). Thereafter approximately 100 µl of 0.1mm Zirconia/Silica glass beads
322 (BioSpec Products, USA) were added and the cultures subjected to four pulses of bead
323 beating using the Roche MagNA Lyser at 7000rpm for 60s, with two minute intermittent
324 incubations on ice. Immediately after bead beating, 200 µl of chloroform was added
325 followed by centrifugation at 15 000 rpm for 15 minutes at 4˚C and separation of the
326 aqueous phase. The RNA was precipitated with 500µl of 100% isopropanol and
327 incubated at -20˚C for 1 hour. After centrifugation at 15 000 rpm for 10 minutes at 4˚C
328 the RNA pellet was washed with 1 ml 75% ethanol, centrifuged at 10 000 rpm for 5
329 minutes at 4˚C and air-dried. The RNA pellet was then dissolved in 30ul of RNase-free
330 water.
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332 ***DNAse treatment and purification.*** The RNA was subjected to DNAse treatment using
333 the DNase I, RNase-free kit (Thermo Scientific, USA), as per manufacturer's
334 instructions. The RNA was then purified using the RNeasy Mini Kit (Qiagen, Germany),
335 during which a second round of DNase digestion took place utilizing the RNase-free
336 DNase Set (Qiagen, Germany). The integrity of the RNA samples was confirmed using
337 an 23S/16S ratio (above ≥ 1.2) determined by an Experion™ Std Sens Analysis Kit (Bio-
338 Rad, USA).
339
340 **RNA sequencing and bioinformatics analysis**
341 ***RNAseq library preparation.*** The Qubit™ RNA Assay kit (Invitrogen, USA) was used
342 with the Qubit®2.0 Fluorometer to quantify the RNA. Following RNA quantification,
343 rRNA was depleted using the Ribo-Zero Magnetic Kit (Illumina, USA). Enriched mRNA
344 was analysed on a RNA specific E-gel EX 2% (Invitrogen, USA) to confirm rRNA
345 removal. After purification of the mRNA using the RNeasy Mini Kit (Qiagen, Germany)
346 RNA sequencing libraries were constructed using the NEBNext® Ultra™ Directional
347 RNA Library Prep Kit for Illumina (New England BioLabs Inc, USA). The prepared
348 libraries were indexed with NEBNext Multiplex Oligos for Illumina (New England
349 BioLabs Inc. USA) and sequenced with 50 bp single end reads on an Illumina HiSeq
350 2000 platform at the Norwegian Sequencing Centre, Oslo, Norway.
351
352 ***Bioinformatics.*** The sequence reads were aligned to the *M. tuberculosis* H37Rv genome
353 (NCBI accession NC_000962.2) using SeqMan NGen from the DNASTAR Lasergene 11
354 software. Transcripts for each sample were quantified and normalized as reads per
355 kilobase per million reads (RPKM). The three replicate RPKM values for each sample



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356 were standardised based on their mean transcript values and were used to assess gene
357 expression and fold change differences between isolates using ArrayStar (DNASTAR).
358 Pairwise comparisons between strains were conducted with confidence intervals and
359 statistics determined using a Students T-test, with multiple testing corrections using the
360 Benjamini Hochberg correction to reduce False Discovery Rate (FDR). Intergenic SNPs
361 present only in the three XDR strains and not in the drug susceptible strain were
362 identified from whole genome sequencing data from a previous study (1). A
363 transcriptomic-genomic analysis was then conducted to identify promoter SNPs
364 associated with at least a 4-fold upregulation of the downstream gene.

365

366 **Whole-genome phylogeny**

367 **Sequence** reads for the four KZN strains were downloaded from SRA (run accessions
368 SRR832991, SRR833024, SRR833121 and SRR924700). Reads were aligned to the
369 H37Rv genome (NC_000962.3) using SeqMan NGen (DNASTAR), resulting in median
370 alignment depths ranging from 184× to 330× for individual isolates. SNPs were called
371 and filtered as previously described.(39) The concatenated SNPs were used to create a
372 distance-based neighbour-joining tree.

373

374 **qRT-PCR**

375 **RNA** was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad,
376 USA). Quantitative Real-Time PCR was conducted using the iTaq™ Universal SYBR®
377 Green Supermix (Bio-Rad, USA) with forward and reverse primers for selected genes of
378 interest. Primers were designed for *ethA*, *inhA, ethR* and a housekeeping gene *sigA*
379 (**Table S5**). Expression levels were normalized to the reference gene *sigA*.



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380

381 **Flow cytometry Promoter Reporter Assay**

382 **To** create a dual colour reporter the Multisite Gateway® Three-Fragment Vector
383 Construction method (Invitrogen, USA) was used. The *ethA*-*ethR* intergenic region,
384 mycobacterial codon optimized Emerald GFP, and mycobacterial codon optimized
385 TagRFP constitutively expressed by the promoter pUV15, were individually cloned into
386 entry vectors. These were combined with a destination vector based on an episomal
387 mycobacterial vector containing a kanamycin resistance cassette (*aph*), mycobacterial
388 origin of replication and *E. coli* origin of replication. Four separate *ethA*-*ethR* intergenic
389 regions were used corresponding to the wild-type and mutant sequences (generated by
390 PCR using genomic DNA from resistant clinical isolates) upstream of *ethA*, and the same
391 pair in the reverse orientation corresponding to the sequences upstream of *ethR* (**Table
392 S6**). Additional plasmids were constructed with the *inhA* promoter with and without an g-
393 17t mutation and a non promoter region (intragenic *katG* sequence) cloned in front of the
394 GFP (**Figure S2**). Promoter sequences for each construct were sequenced confirmed.
395 Respective plasmids were transformed into H37Rv using standard protocols.(40)

396

397 **Strains** harbouring the dual-colour reporters were grown up to mid-log phase ($OD_{600}$ of
398 0.5 - 0.8) in 7H9 media containing 25mg/L kanamycin. 1mL of each strain was then
399 filtered through a 10 micron filter and acquired on the BD FACS Aria III using BD DIVA
400 software. 100 000 events were recorded with single cell acquisition set at a threshold rate
401 of ~ 5000-7000 events per second. Green and red fluorescence were detected using the
402 FITC and PI filters respectively. The gating strategy employed during acquisition and
403 software analysis, using FlowJo V10, differentiated single cells/events based on the

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404 relationship between cell size (forward scatter – FSC) and granularity (side scatter –
405 SSC). Secondary gating was done using FlowJo on events with red fluorescent signal to
406 ensure only cells containing expression vectors were included in our analysis. Median
407 fluorescent intensity (MFI) of red and green fluorescent signals were extracted. MFI of
408 green fluorescence was normalized to MFI of red fluorescence for each replicate before
409 calculating mean and standard deviation. A two sided t-test was used to determine
410 statistical significance.

411

412 **Drug susceptibility testing**

413 **100ul** of three dilutions of each strain, including 1 x$10^6$, 1 x$10^4$ and 1 x$10^3$ cells, were
414 plated out onto quadrant plates containing BD Difco™ Middlebrook 7H10 agar with
415 varying drug concentrations of ethionamide (1.25, 2.5, 5, 10, 20, 40 and 80mg/L) and
416 counted for CFU after 3 weeks incubation at 37°C.

417

418 **Global Distribution of *ethA* promoter mutations**

419 **A** global dataset of 5310 *M. tuberculosis* strains from five continents (28) was searched
420 for all instances of *ethA* promoter mutations. To identify individual mutation arisal
421 events across the phylogeny, we performed parsimony-based analysis using the PAUP
422 software, version 4.0b10 (29) as in Manson et al (28).

423

424 **FUNDING INFORMATION**

425 **This** project received funding from the Africa Health Institute (AHRI) and has also been
426 funded in part with Federal funds from the National Institute of Allergy and Infectious
427 Diseases, National Institutes of Health, Department of Health and Human Services under

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428 Grant No. U19AI110818, and Contract No. HHSN272200900018C to the Broad Institute.
429 The funders had no role in study design, data collection and interpretation, or the decision
430 to submit the work for publication.

431

432 **ACKNOWLEDGEMENTS**

433 **We** would like to acknowledge and thank M.R Farhat and M. Murray for providing us
434 with additional information on previous work they had conducted surrounding the *ethA-*
435 *ethR* locus, A.R Baulard for his helpful comments and suggestions, K.A Cohen for her
436 insights and information regarding the clinical strains used in this study and V.
437 Munsamy-Govender for her assistance in locating and propagating some of these strains.

438

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602
603 **FIGURE LEGENDS**

604 **FIG 1:** (A) Phylogenetic tree representing the distribution of the 4 strains (shown in red
605 and boxed) selected for RNAseq. (B) Hierarchical gene clustering of these strains, based
606 on their relative gene expression, shows that the drug susceptible strain clusters
607 separately from the others. (C) Venn diagram representing genes differentially expressed
608 7-fold or greater relative to the susceptible control. The blue, red and green circles
609 represent pairwise comparisons with TKK-01-0033, TKK-01-0025 and TKK 01-0040
610 respectively
611

612 **FIG 2:** Representation of the intergenic region between *ethA* and *ethR*. The location of
613 the single nucleotide polymorphism (SNP) is found 11 base pairs upstream of *ethA* and is
614 indicated in red. The *ethR* binding region is indicated by the black box (25)
615

616 **FIG 3:** Analysis of promoter activity between wild type and mutant constructs. The left
617 panels represent the ratio of the mean fluorescence intensity (MFI) of green fluorescent
618 protein (GFP) to red fluorescent protein (RFP), as well as statistical differences between
619 the wild type and mutant constructs for (A) *inhA* promoter and (B) *ethA* and *ethR*



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620 promoters. p - values are indicated on the bar charts. The panels on the right represent
621 single cell counts from flow cytometry. RFP expression is represented on the y-axis as
622 the PI-H channel and GFP expression is represented on the x-axis as the FITC-H channel.
623
624 **FIG 4:** Relative gene expression of *ethA, ethR* or *inhA* in clinical strains of *M.*
625 *tuberculosis* (Table S5). Gene expression levels were normalised to *sigA* for each strain.
626 Relative normalised expression represents the fold change in normalised expression of
627 each strain compared to the drug susceptible clinical strain 84. Light blue bars represent
628 strains that do not contain t-11c *ethA* promoter mutations and dark blue bars represent
629 strains that have the t-11c *ethA* promoter mutation. Light pink bars represent strains
630 without *inhA* promoter mutations and dark pink bars represent strains with *inhA* promoter
631 mutations. TKK strain numbers are abbreviated to their last two digits. E.g. 62 represents
632 TKK-01-0062. Statistical significance of relative normalised expression for *ethA* and
633 *inhA* was derived using unpaired t-tests between each strain and the clinical drug
634 susceptible strain 84 (shown in black). In addition, statistical significance of relative
635 normalised expression for *ethA* was derived using unpaired t-tests between each strain
636 and strain 62, which does not harbour a t-11c *ethA* promoter mutation (shown in red).
637 1551 corresponds to the laboratory strain CDC1551 and was excluded from this analysis.
638 ** = p-value $\leq$ 0.01, * = p-value $\leq$ 0.05, NS = not significant
639
640 **TABLE 1:** Strain details including resistance mutations and RNA sequencing coverage
641
642 **TABLE 2:** Minimal inhibitory concentrations (MICs) of clinical strains to ethionamide
643 (ETH)

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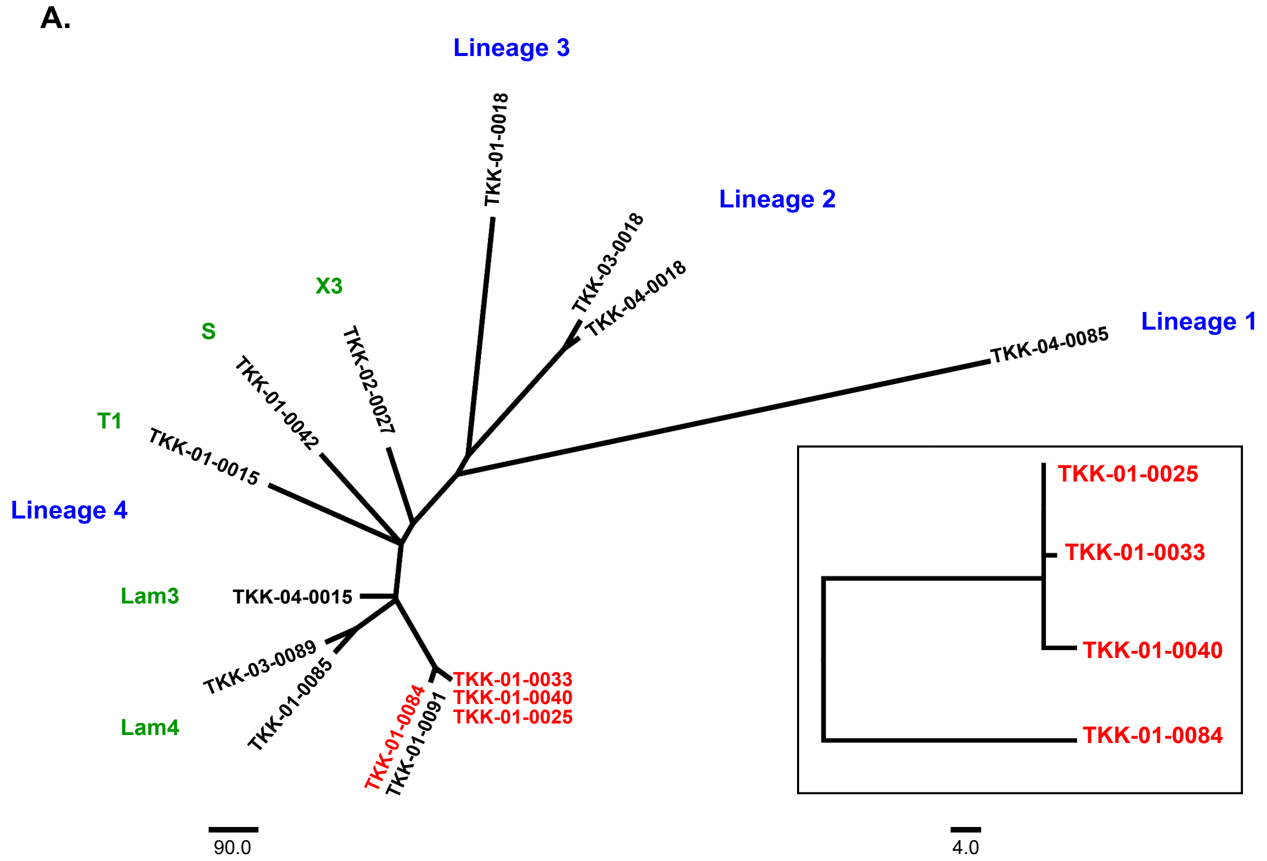
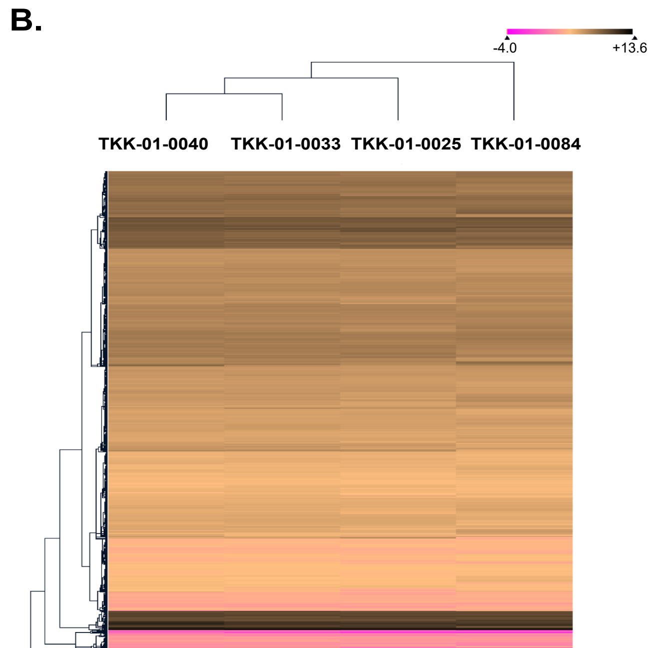
FIG 1



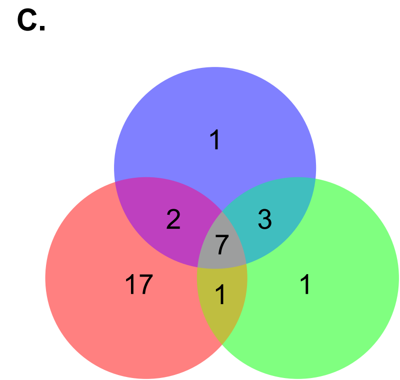




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FIG 2

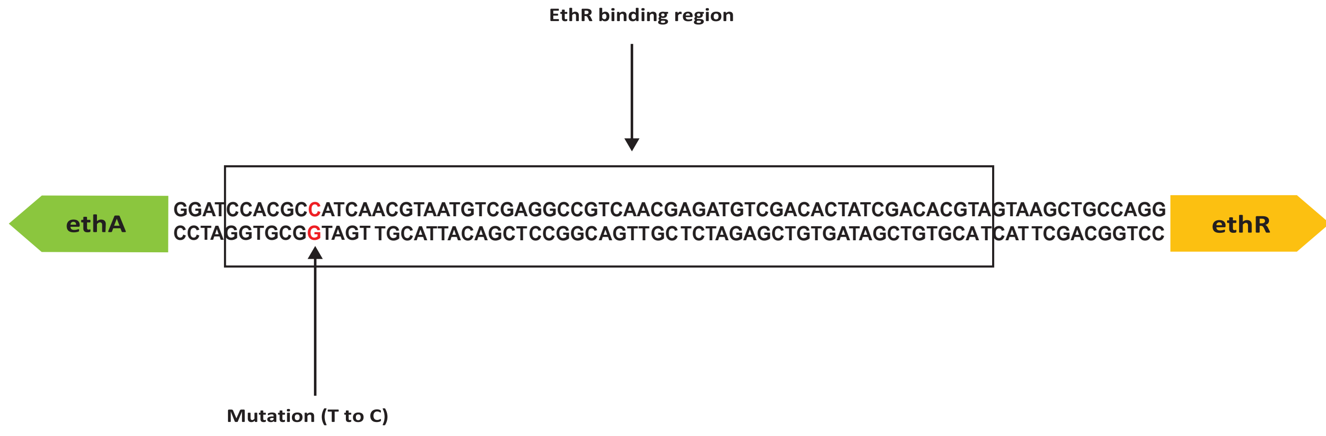


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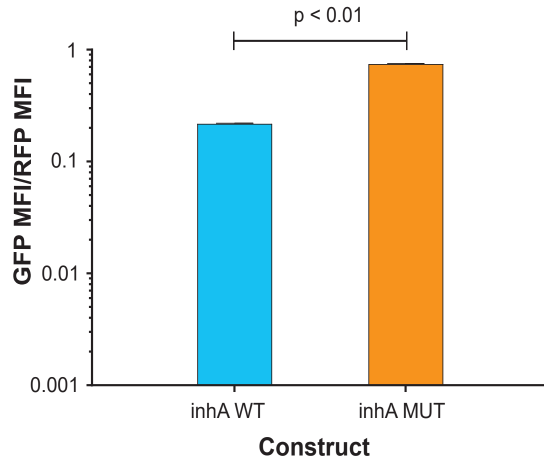
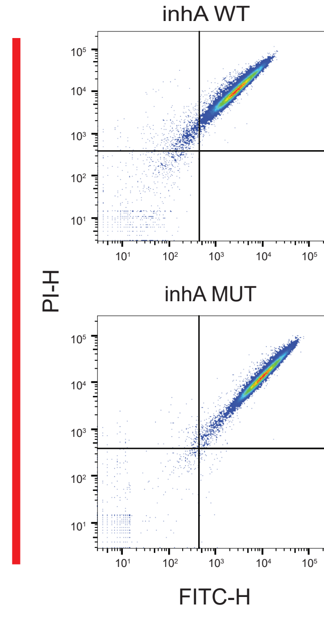
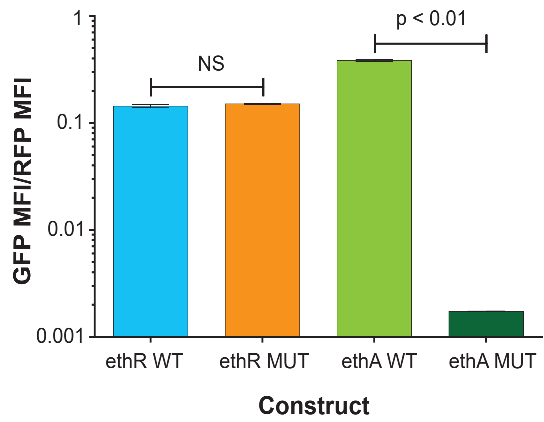
FIG 3

**A.**




**B.**



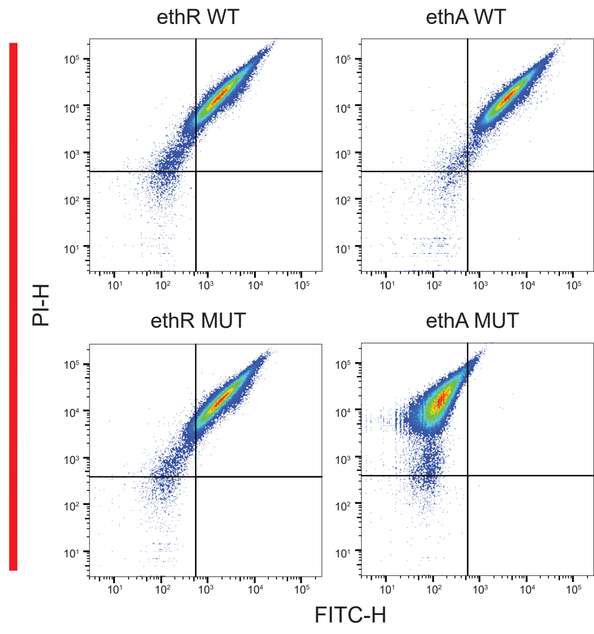




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FIG 4

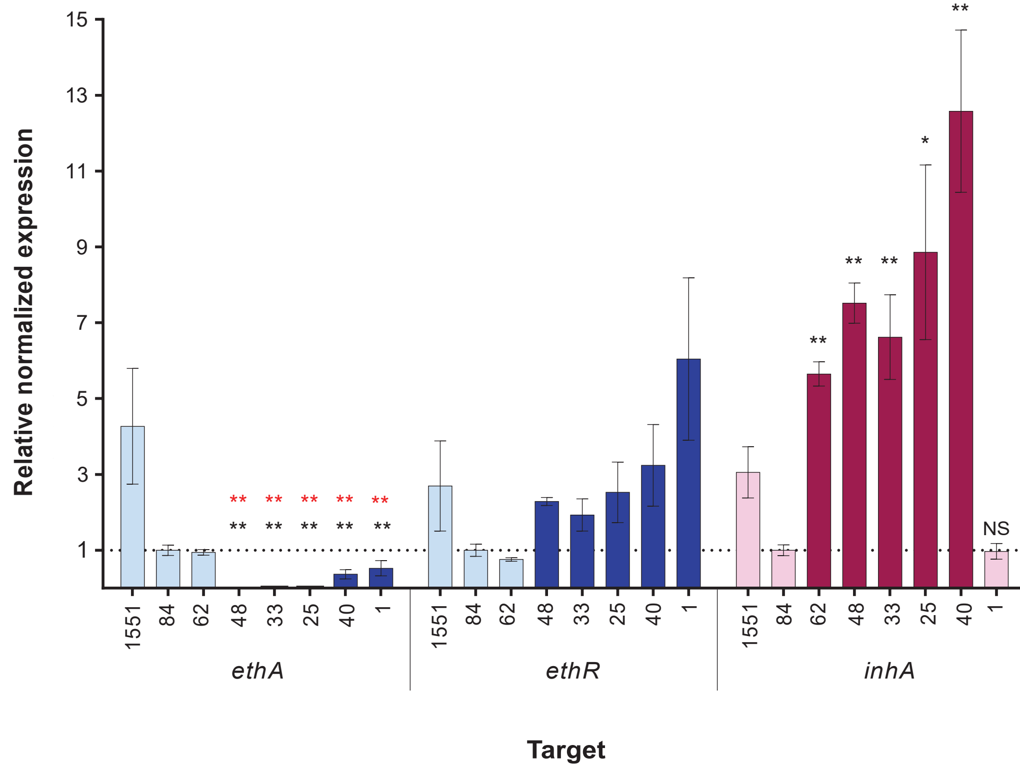


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**TABLE 1** Strain details including resistance mutations and RNA sequencing coverage

| Strain | Spoligotype | Resistance mutations for each corresponding drug | | | | | | | RNAseq Coverage (x) |
|---|---|---|---|---|---|---|---|---|---|
| | | INH | RIF | STR | EMB | KAN | ETH | OFL | |
| TKK-01-0084 | LAM4 | | | | | | | | 288.63 |
| TKK-01-0025 | LAM4 | *inhA* t-8a<br>*katG*:S315T | *rpoB*:L452P<br>D435G | *gidB*:L16R<br>*gidB*:del | *embB*:M306V | *rrs*:A1401G | *inhA* t-8a | *gyrA*:A90V | 214.34 |
| TKK-01-0033 | LAM4 | *inhA* t-8a<br>*katG*:S315T | *rpoB*:L452P<br>D435G | *gidB*:L16R<br>*gidB*:del | *embB*:M306V | *rrs*:A1401G | *inhA* t-8a | *gyrA*:A90V | 239.13 |
| TKK-01-0040 | LAM4 | *inhA* t-8a<br>*katG*:S315T | *rpoB*:L452P<br>D435G | *gidB*:L16R<br>*gidB*:del | *embB*:M306V | *rrs*:A1401G | *inhA* t-8a | *gyrA*:A90V | 269.24 |

INH = Isoniazid, RIF = Rifampicin, STR = Streptomycin, EMB = Ethambutol, KAN = Kanamycin, ETH = Ethionamide, OFL = Ofloxacin

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**TABLE 2** Minimal inhibitory concentrations (MICs) of clinical strains to ethionamide (ETH)

| Strain | DST | Spoligo | Putative ETH resistance conferring mutations | | | | | | | | MIC (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *ethA* promoter | *inhA* promoter | *inhA intragenic* | *ethA* intragenic | *ethR* intragenic | *mymA* | *mshA* | *ndh* | |
| TKK-01-0001 | MDR | KZN | t-11c | | | | | | | | 20 |
| TKK-01-0035 | MDR | KZN | t-11c | | | | | | | | 10 |
| TKK-01-0075 | MDR | KZN | t-11c | | | | | | | | 5 |
| TKK-01-0025 | XDR | KZN | t-11c | t-8a | | | | | | | 80 |
| TKK-01-0040 | XDR | KZN | t-11c | t-8a | | | | | | | > 80 |
| TKK-01-0048 | MDR | KZN | t-11c | t-8a | | | | | | | 20 |
| TKK-01-0033 | XDR | KZN | t-11c | t-8a | | | | | | | 80 |
| TKK-02-0001 | XDR | BEIJING | | c-15t | I194V | | P94P | | A189T | | 40 |
| TKK-02-0046 | POLY (P/N + RIF) | BEIJING | | c-15t | I194V | | P94P | | A189T | | 20 |
| TKK-01-0005 | POLY (STR+ETH) | BEIJING | | c-15t | | | | | A189T | | 80 |
| TKK-01-0062 | PXDR | BEIJING | | g-17t | | A381P | | | | | 20 |
| TKK-01-0032 | MDR | S | | t-8g | | | | | | V18A | 5 |
| TKK-01-0013 | XDR | BEIJING | | | | Y276H | | | A189T | | 5 |
| TKK-02-0018 | MDR | T3 | | | | V202L | | del | | | 15 |
| TKK-02-0019 | PXDR | | | | | V202L | | del | | | 20 |
| TKK-01-0026 | MDR | T3 | | | | | | del | | | 2,5 |
| TKK-02-0069 | PXDR | | | | | | | del | | | 5 |
| TKK-01-0081 | DS | KZN | | | | | | | | | 2,5 |
| TKK-01-0084 | DS | KZN | | | | | | | | | 2,5 |
| TKK-01-0047 | DS | BEIJING | | | | | | | | | 2,5 |
| TKK-01-0027 | DS | BEIJING | | | | | | | | | 2,5 |
| H37Rv | DS | n/a | | | | | | | | | 2,5 |
| CDC 1551 | DS | n/a | | | | | | | | | 1,25 |

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