

1 **Characterization of genomic variants associated with resistance to bedaquiline and**
2 **delamanid in naïve *Mycobacterium tuberculosis* clinical strains**

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31 **Running title:** Markers of resistance to bedaquiline and delamanid.

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33 **Abstract**

34 The role of genetic mutations in genes associated to phenotypic resistance to bedaquiline
35 (BDQ) and delamanid (DLM) in *Mycobacterium tuberculosis* complex (MTBc) strains is
36 poorly understood. However, a clear understanding of the role of each genetic variant is
37 crucial to guide the development of molecular-based drug susceptibility testing (DST). In
38 this work, we analysed all mutations in candidate genomic regions associated with BDQ-
39 and DLM-resistant phenotypes using a whole genome sequencing (WGS) dataset from a
40 collection of 4795 MTBc clinical isolates from six high-burden countries of tuberculosis
41 (TB). From WGS analysis, we identified 61 and 158 unique mutations in genomic regions
42 potentially involved in BDQ- and DLM-resistant phenotypes, respectively. Importantly, all
43 strains were isolated from patients who likely have never been exposed to the medicines.
44 In order to characterize the role of mutations, we performed an energetic *in silico* analysis
45 to evaluate their effect in the available protein structures Ddn (DLM), Fgd1 (DLM) and
46 Rv0678 (BDQ), and minimum inhibitory concentration (MIC) assays on a subset of MTBc
47 strains carrying mutations to assess their phenotypic effect. The combination of structural
48 protein information and phenotypic data allowed for cataloging the mutations clearly
49 associated with resistance to BDQ ($n= 4$) and DLM ($n= 35$), as well as about a hundred

50 genetic variants without any correlation with resistance. Importantly, these results show
51 that both BDQ and DLM resistance-related mutations are diverse and distributed across
52 the entire region of each gene target, which is of critical importance to the development of
53 comprehensive molecular diagnostic tools.

54 **Importance**

55 Phenotypic drug susceptibility tests (DST) are too slow to provide an early indication of
56 drug susceptibility status at the time of treatment initiation and very demanding in terms of
57 specimens handling and biosafety. The development of molecular assays to detect
58 resistance to bedaquiline (BDQ) and delamanid (DLM) requires accurate categorization of
59 genetic variants according to their association with phenotypic resistance. We have
60 evaluated a large multi-country set of clinical isolates to identify mutations associated with
61 increased minimum inhibitory concentrations (MICs) and used an *in silico* protein structure
62 analysis to further unravel the potential role of these mutations in drug resistance
63 mechanisms. The results of this study are an important source of information for the
64 development of molecular diagnostic tests to improve the provision of appropriate
65 treatment and care to TB patients.

66 **1. Introduction**

67 The management of drug resistant tuberculosis (DR-TB) caused by *Mycobacterium*
68 *tuberculosis* complex (MTBc) strains poses a serious public health challenge worldwide.
69 The World Health Organization (WHO) new guidelines recommend the use of bedaquiline
70 (BDQ) for all TB cases of rifampicin resistance (RR-TB), multi drug resistant (MDR) TB
71 (MTBc strains resistant at least to isoniazid and rifampicin) and extensively drug-resistant
72 (XDR) TB (MDR strains resistant to fluoroquinolones and second-line injectables drugs)
73 (1). Based on WHO priority grouping of medicines, Delamanid (DLM) compound is
74 recommended when an effective regimen cannot be established by group A agents,

75 containing BDQ, fluoroquinolones (FQs), linezolid (LZD) and B agents group, composed
76 by clofazimine (CLF) and cycloserine (CS) (2). Previous studies have shown that
77 MDR/XDR-TB patients treated with BDQ and DLM rapidly develop resistance due to fixed
78 mutations in candidate genes which often appear as previously undescribed novel variants
79 (3, 4, 5, 6, 7). Additionally, resistance to BDQ can arise in naïve populations of MTBc
80 strains as a consequence of a clofazimine-containing regimen, or by random mutations
81 affecting the drug targets (8, 9, 10, 11, 12, 13, 14). DLM resistance not associated to
82 exposure has been reported as well (15, 16, 17, 18, 19). Moreover, as a member of the
83 nitroimidazooxazines, DLM shares the same resistance mechanisms of pretomanid (PA-
84 824) compound (20).

85 Therefore, knowledge of the BDQ and DLM susceptibility status of clinical MTBc isolates
86 before therapy has started and the early detection of emerging resistance in failing
87 regimens are needed to ensure an effective treatment of DR-TB.

88 Here, whole genome sequencing (WGS) based approaches, that are rapidly expanding
89 from basic research into routine diagnostic laboratories, provide the advantage of
90 interrogate virtually all resistance targets in a given clinical MTBc strain. However, the
91 routine diagnostic application of WGS requires a much better understanding of the
92 correlation between genotypic and phenotypic, particularly for the new drugs (21, 22).

93 Currently, the molecular mechanisms leading to resistance to BDQ and DLM are not well
94 described, a fact that jeopardises the design of a reliable molecular approach to detect
95 resistance.

96 Mutations in *atpE* (*Rv1305*), which encodes for the C-subunit of ATP synthase, have been
97 associated with phenotypic resistance to BDQ, which is known to directly inhibit the ATP
98 synthase (on target mechanism) (23). In addition, the mutations in the off-target *Rv0687*
99 gene result in increased minimum inhibitory concentrations (MICs) for BDQ by up-

100 regulation of the MmpL5/MmpS5 pump gene expression, concurrently leading to a cross-
101 resistance to clofazimine (CLF) (**10, 11, 24**). Furthermore, it has been demonstrated that
102 mutations in *pepQ* (*Rv2535c*) may confer low-level resistance to both CLF and BDQ in
103 clinical isolates (**9, 25**).

104 DLM impairs the biosynthesis of mycolic acids and requires activation by the F420-
105 dependent nitroreductase encoded by the *ddn* gene (on target mechanism). The F420
106 cofactor is synthesized by enzymes encoded by the *fbiA*, *fbiB*, *fbiC*, *fgd1* genes, all of
107 which are involved in DLM off-target mechanisms. Polymorphisms in these genes were
108 shown to be involved in phenotypic DLM resistance (**26, 27**).

109 To better describe BDQ and DLM resistance mechanisms, we investigated the genomic
110 regions involved in resistance to BDQ and DLM from 4795 MTBc isolates collected within
111 a multi-country drug-resistance surveillance study and to identify variants potentially
112 involved in resistance development (**28**). The mutations found were correlated with strain
113 lineage, DR-profile and country of origin. To combine the genomic data with phenotypes,
114 we performed BDQ and DLM MICs for a subset of these isolates. Finally, for the available
115 3D protein structures (Ddn, Fgd1 and Rv0678) we performed an *in silico* structural and
116 energetic analysis in order to characterize and quantify the mutation effect on protein
117 function. This combined information enabled us to provide a first robust catalogue of BDQ
118 and DLM resistance mutations as basis of the establishment of WGS resistance prediction
119 algorithms for these drugs.

120 **2. Methods**

121 **2.1 Study design**

122 A total of 4795 genome sequences retrieved from the Sequence Read Archive of the
123 National Center for Biotechnology Information as recalibrated BAM files (accession
124 number SRP128089) were considered and investigated in this study. The corresponding

125 MTBc isolates originate from a unique population-based surveillance study across six
126 countries with a high burden of TB or MDR-TB, according to WHO's high burden country
127 list for the period of 2016-2020: Azerbaijan ($n = 751$), Belarus ($n = 197$), Bangladesh
128 ($n = 935$), Pakistan ($n = 194$), South Africa ($n = 1578$), Ukraine ($n = 1140$) (**28**). For our
129 purposes, all sequenced isolates harbouring at least one single nucleotide polymorphism
130 (SNP) or insertion/deletion (indel) in at least one of the candidate genomic regions for DLM
131 and/or BDQ resistance were considered for the analysis, excluding synonymous mutations
132 and previously characterized lineage-associated SNPs for which the absence of
133 correlation with the phenotypic DLM resistance was demonstrated (**15, 16**). For genetic
134 variants detected in more than one isolate we decided to replicate results selecting two
135 isolates from different countries, whenever possible. The flowchart for sample selection,
136 the number of isolate tested and phenotypic drug-susceptibility testing (DST) of selected
137 isolates are reported in Figure S1 of supplementary materials.

138 **2.2 Whole Genome Sequencing analysis**

139 WGS data were generated by both Illumina technology (Illumina, San Diego, CA, USA)
140 and Ion Torrent technology (ThermoFisher Scientific) as previously described (**28**).
141 Sequencing data were analysed using the MTBseq pipeline (**29**) to identify all variants in
142 the genomes and MTBc lineage. The analysis was performed on the mapped MTBc reads
143 by setting a quality threshold of at least a mean coverage of 20x and an unambiguous
144 base call threshold of $\geq 70\%$. A mutation was called only if SNPs and/or indel variants were
145 detected by at least eight reads (both forward and reverse reads) with a minimum phred
146 quality score of 20, and by considering a mutation frequency of $\geq 75\%$. The regions of the
147 MTBc genome reference H37Rv NC_000962.3 (**30**) considered in the study are reported
148 in Table S1 of supplementary materials. The WGS analysis results and distribution of

149 mutations among lineages and countries of isolation are reported in the supplementary
150 excel Dataset S1.

151 Cluster analysis was performed on the distance matrix generated by the MTBseq pipeline
152 using *in-house* python scripts (<https://github.com/aspitaleri/python>). The distance matrix
153 was analysed using a hierarchical linkage clustering method with a 12 SNPs cut-off (31).

154 **2.3 Minimum Inhibitory Concentration assay**

155 The selected MTBc isolates for genetic variants were sub-cultured on Löwenstein-Jensen
156 medium and subsequently subjected to MIC testing against BDQ and/or DLM by the
157 resazurin colorimetric microtiter plate assay (REMA) as previously described (16, 32, 33).
158 Delamanid powder was obtained from Otsuka Pharmaceutical (Tokyo, Japan) and pure
159 bedaquiline powder was obtained from Janssen Pharmaceutical (Beerse, Belgium). A
160 DLM concentration range of 0.004-4 µg/ml and a BDQ concentration range of 0.004-2
161 µg/ml were used, considering the proposed cut-off values of 0.12 µg/ml and 0.06 for BDQ
162 and DLM, respectively (34). Based on MIC results, the isolates were categorized as
163 susceptible (S; MIC ≤ cut-off), low resistant level (I; MIC 1 dilution > cut-off) or resistant (R;
164 MIC more than 1 dilution > cut-off). All MIC values reported in this work correspond to the
165 MIC₁₀₀ value that considers any change in colour to purple/pink as indicating the
166 presence of viable bacilli (33). For each batch of isolates tested, the H37Rv *M.*
167 *tuberculosis* reference strain (*M. tuberculosis* H37Rv ATCC 27294) was included as a
168 control, and test isolate results of that batch were accepted only if the H37Rv MIC value
169 was within the expected range of ≤0.004-0.03 µg/ml for DLM and ≤0.008-0.03 µg/ml for
170 BDQ. Further details of REMA protocols are reported in supplementary materials Text S1
171 word file.

172 **2.4 Mutation structural analysis**

173 We carried out an energetic analysis on the available crystal structures of proteins Ddn
174 (PDB 3R5R), Fgd1 (PDB 3B4Y) and Rv0678 (PDB 4NB5) (35, 36, 37) using Eris (38) and
175 MAESTRO (39) programs. We exploited two end-point methods to evaluate the change of
176 protein stability upon mutations, namely Eris and MAESTRO, which calculate the folding
177 free energy in two different manners. For this structural analysis stop codons, frameshifts,
178 and SNPs affecting the promoter region were not included. The stability change, $\Delta\Delta G$, is
179 computed as the difference between the average stabilities of mutant and wild type protein
180 structures. Both *in silico* approaches were used as a qualitative cross-validation to
181 evaluate the protein mutation effects, considering 0.34 Kcal/mol and 5 Kcal/mol as
182 thresholds for MAESTRO and Eris, respectively.

183 In addition to folding stability, we calculated the effect of mutations on the complex
184 stabilities Ddn-F420-H₂ Fgd1-F420-H₂ using DSX pair potentials knowledge-based
185 scoring function (40). In case of Rv0678 protein we also performed an energetic analysis
186 to quantify the effect of the mutations on the homodimer protein-protein stability. For this
187 purpose, we carried out a molecular mechanics energies combined with the Generalized
188 Born and surface area continuum solvation (MM/GBSA). The calculation was performed
189 using MMPBSA.py program within Amber14 suite using ff14 force field and the GB^{OBC1}
190 implicit solvent model (41). All obtained *in silico* results are reported in the supplementary
191 material Dataset S2. Further details of *in silico* analyses are reported in supplementary
192 materials Text S1 word file. Primary protein sequences alignment for the frameshift
193 analysis was performed using ClustalX algorithm (42). The visualization of the mapped
194 mutations on the protein structures are created with PyMOL v2.0 (43).

195 **3. Results**

196 A total of 4795 WGS from MTBc clinical strains were analysed by considering the
197 candidate genomic regions for BDQ resistance (*atpE*, *Rv0678*, *pepQ*) and for DLM

198 resistance (*ddn*, *fgd1*, *fbiA*, *fbiB*, *fbiC*). This collection included 731 (17%) MDR and 79
199 (2%) XDR MTBc strains (Fig. S1). Based on WGS results, we identified a total of 106 and
200 643 isolates harboring relevant genomic variants potentially involved in BDQ and DLM
201 resistance, respectively. We tested a subset of isolates carrying mutations for phenotypic
202 DST for BDQ ($n=51$) and for DLM ($n=124$) representing the 43 and 104 BDQ- and DLM-
203 related variants, respectively (Fig. S1). All genomic variants detected by WGS analysis in
204 candidate genes for BDQ and DLM with the corresponding information of MTBc strain
205 lineage, drug resistance profile, country of isolation, mutation frequency and MIC results
206 for tested MTBc isolates are reported in Dataset S1 of supplementary material.

207 **3.1. Analysis of mutations for BDQ resistance**

208 The WGS analysis revealed 61 unique mutations in the considered genomic regions
209 associated with BDQ resistance (Fig. S1). The mutation analysis distribution revealed 27
210 unique mutations in *Rv0678* (including 7 mutations in the promoter region, 16
211 nonsynonymous mutations, and 4 indels causing frameshift mutations), 32 unique
212 mutations in the *pepQ* gene (including 2 upstream mutations, 28 nonsynonymous
213 mutations and 2 frameshift mutations), and 2 upstream mutations in the *atpE* gene, while
214 no mutations were found in the *atpE* encoding region (Dataset S1).

215 Phenotypic testing revealed that four different *Rv0678* mutations had MIC values above
216 the cut-off of 0.12 $\mu\text{g/ml}$: two frameshift (fs) mutations in *Rv0678*, Gly6fs (del_16-17 gg)
217 and the double mutant Gln9fs-Thr92fs (ins_27 c, ins_274 a) associated with an MIC of 0.5
218 $\mu\text{g/ml}$, and two *Rv0678* nonsynonymous mutations Arg96Trp and Met111Lys yielding a
219 low level of resistance to BDQ of 0.25 $\mu\text{g/ml}$ (Table 1). The two frameshift mutations
220 associated with BDQ-resistant phenotypes were observed in one MDR isolate and another
221 MDR isolate with concurrent resistance to FQs (corresponding to one new and one

222 previously treated TB case, respectively). Two nonsynonymous mutations associated to
223 low level resistance were found in two pan-susceptible (full-S) MTBc strains (Table 1).
224 The other 39 mutations were detected in isolates susceptible to BDQ with MIC values
225 ≤ 0.12 $\mu\text{g/ml}$, including two isolates harbouring frameshifts in Rv0678: Ile16fs (ins_ 46
226 tcatggaattcg) and Ala153fs (ins_457 c) showing an MIC of 0.06 $\mu\text{g/ml}$. (Dataset S1). The
227 protein amino acid sequences obtained from these two frameshifts mutations were aligned
228 to the Rv0678 wild-type sequence, highlighting that both wild-type and mutated proteins
229 contain the two well-conserved and important regions: the amino acid (aa) positions from
230 34 to 99 (DNA-binding domain) and positions from 16 to 32 and from 101 to 160
231 (dimerization domains) (Fig. 1). In the case of *Rv0678* Ile16fs, the insertion of 12
232 nucleotides caused the addition of 4 aa from position 16 of the Rv0678 protein without
233 disrupting the frame of the whole enzyme, while the Ala153fs caused a change to the last
234 13 aa of the C-terminal of the protein (Fig 1). This suggests that these frameshifts do not
235 affect protein stability and function resulting in the BDQ-susceptible phenotype.
236 A structural analysis of the effect of mutations in Rv0678 resulting in amino acid changes
237 was performed as previously described by the Eris and MAESTRO computational
238 approaches (Dataset S2). The Rv0678 folding stability calculation by ERIS software
239 showed that both Arg96Trp and Met111Lys mutations associated with the BDQ-resistant
240 phenotype altered Rv0678 protein folding/stability ($\Delta\Delta\text{G kcal/mol} > 5$). These two mutations
241 are localized in the dsDNA-binding and dimerization domain regions of Rv0678,
242 respectively (Fig. 2). Interestingly, the Met111Val mutation had a milder effect on the
243 protein stability than Met111Lys which is reflected in the low BDQ MIC value. Both Eris
244 and MAESTRO analysis showed that the other mutations in Rv0678 have a lower
245 estimated effect on protein stability which is in accordance with lower MIC values for these
246 clinical strains (Dataset S2). As these approaches do not consider the effect of the

247 mutations on dimerization protein function, we calculated the protein-protein binding free
248 energy under the MM/GBSA approximation for the mutations which localize in the Rv0678
249 dimerization domain (Fig. 2). The results showed that five mutations (Met111Lys,
250 Leu117Arg, Val120Met, Asp141His and Met146Arg) have a significant increase of $\Delta\Delta G$
251 kcal/mol in the protein-protein homodimer binding free energies, indicating that they can
252 affect the dimerization process, destabilizing the overall homodimer stability (Dataset 2).
253 This data suggest that these mutations could be directly involved in the slight increase in
254 BDQ MIC for these strains, all with a BDQ MIC of 0.12 $\mu\text{g/ml}$ except for the MTBc strain
255 harboring the *Rv0678* Met146Arg variant with a BDQ MIC of 0.06 $\mu\text{g/ml}$. (Fig. 2).

256 An analysis of correlation between observed mutations in *Rv0678*, *atpE* and *pepQ* regions
257 with lineage and country of origin, revealed that the majority of mutations ($n= 46$; 75.4%)
258 occurred only once (Fig. 3). Only four mutations, all in *Rv0678* gene, were detected in
259 more than 5 isolates, all of them showing a BDQ-susceptible phenotype: the a-4t mutation
260 in the promoter region of *Rv0678* was found in 12 Beijing (2,2,1) isolates from Azerbaijan,
261 the Val3Ile mutation in 8 LAM (4,3,4,2) isolates from Bangladesh, Asn4Thr in 6 Delhi-CAS
262 (3) isolates from Pakistan and Gly87Arg in 8 EAI (1,1,2) isolates from Bangladesh and
263 Pakistan (Fig. 3).

264 Looking at the BDQ-resistance associated variants, the two frameshift mutations
265 associated with high level of BDQ MICs were both observed in isolates from Pakistan and
266 belonged to EAI (1,1,2) and Delhi-CAS (3) lineages, while the two *Rv0678* mutations
267 associated with low level of BDQ resistance, Arg96Trp and Met111Lys, were both
268 observed in two isolates from Bangladesh belonging to Delhi-CAS (3) and Haarlem
269 (4,2,1,2) lineages (Fig. 3).

270 **3.2. Analysis of mutations for DLM resistance**

271 The 643 MTBc isolates identified by WGS as harbouring at least one mutation in one of
272 the candidate genes for DLM resistance represented 164 unique DLM-related mutations.
273 The WGS analysis revealed 30 unique mutations in *ddn* (including 3 nonsense and 2
274 frameshift mutations), 25 unique mutations in *fbiA* (including 1 nonsense mutation), 23
275 unique mutations in *fbiB*, 65 unique mutations in *fbiC* (including 2 frameshift mutations)
276 and 24 unique mutations in *fgd1* gene (Dataset S1).

277 Considering all unique mutations, twenty (12.2%) were combinations of two or three
278 variants in more than one candidate gene. Phenotypic results revealed that out of the 124
279 isolates tested for DLM, 26 (21%) were resistant to DLM, 13 (10.5%) showed a low level of
280 resistance (MIC = 0.12 µg/ml), while 85 (68.5%) were DLM susceptible (Dataset S1). The
281 DLM-resistant isolates spanned 32 different mutations (Table 2). Considering the
282 phenotypic drug resistance profiles of the DLM-resistant isolates, only six were MDR-TB
283 strains, five of which were retreatment TB cases and one was a new TB case. The
284 analysis of mutation types associated with DLM-resistant revealed 3 nonsense mutations
285 leading to truncated proteins, 3 frameshift mutations and 26 nonsynonymous mutations
286 leading to a single amino acid change (Table 2).

287 Overall, the MIC levels among DLM-resistant isolates ranged from 0.12 µg/ml to ≥4 µg/ml,
288 with the highest MIC values occurring with mutation types causing a truncated Ddn or FbiA
289 protein (frameshift or stop codon mutations). The remaining mutations were associated
290 with increased DLM MIC values between 0.12 and 0.5 µg/ml. Opposite to the high MIC
291 level (≥4 µg/ml) observed for the frameshift at codon 14 in the *ddn* gene, the observed
292 frameshift at the very end of the *fbiC* gene (codon 855) caused a lower increase in MIC
293 level at 0.5 µg/ml (Table 2).

294 Similar to Rv0678, the mutation structural analysis was performed for the Ddn (PDB 3R5L;
295 3R5R) and Fgd1 (PDB 3B4Y; 3C8N) proteins (Dataset S2). The highly conserved Ddn

296 protein catalyzes the reduction of nitroimidazoles of DLM prodrug by the co-factor F420-
297 H₂, resulting in intracellular release of lethal reactive nitrogen species (36). For the on-
298 target Ddn protein, mutations Asn91Thr and Pro86Thr localize very close to the cofactor
299 binding site (Fig 4A) and Δ DSX energies resulted from DrugScore analysis indicate that
300 these are the only two mutations which reduce binding affinity between Ddn and the
301 cofactor F420-H₂ (Dataset S2). The Ddn mutation Thr140Ile is far from the cofactor
302 binding site but its effect on MIC increase is due to the protein folding stability, because
303 the side chain of Thr140 residue is involved in the hydrogen bond network with Ala82-
304 Lys79-F420-H₂ (Fig. 4A). The mutation Val61Gly, which has a mild effect on the MIC (0.12
305 μ g/ml), showed high levels of $\Delta\Delta$ G Kcal/mol with both the Eris and MAESTRO analyses,
306 suggesting a role in destabilizing the folding protein in the β -sheet (Fig. 4A). The analysis
307 of point mutations in Ddn without available MIC values revealed a high level of $\Delta\Delta$ G
308 Kcal/mol energy for mutations Arg72Gln, Pro86Thr and Glu150Ala, suggesting their
309 potential involvement on Ddn stability and consequently phenotypic DLM resistance
310 (Dataset S2).

311 The DLM off-target F420-dependent glucose-6-phosphate dehydrogenase (Fgd1) is
312 important in MTBc energy metabolism, and it is implicated in DLM redox processes related
313 to non-replicating persistence by providing the reduced co-factor F420-H₂ (35). The
314 reported MIC values did not show a strong effect in the *in vitro* experiments because all
315 identified mutations are further than 10 Å from the co-factor F420 binding site (Fig. 4B).
316 Moreover, the computational Eris approach predicts that two Fgd1 mutations without
317 phenotypic data, Ala27Gly and Val165Leu, have potential roles in protein destabilization
318 (Dataset S2). The Fgd1 mutation Gly314Glu which was observed in a strain with only a
319 moderate increase of DLM MIC level seemed to poorly correlate with DLM phenotype,

320 suggesting that other factors could contribute to this small variation of the DLM MIC level
321 in MTBc strains.

322 The distribution analysis of mutations across genotypes and country of isolation showed
323 that *ddn* mutations involved in a DLM-resistant phenotype are represented only once or
324 twice. Exception are, Pro2Gln which was found in seven mainly-T (4,8) isolates (all from
325 Azerbaijan), Asn91Thr in *ddn* in combination with mutation Val58Ile in *fbiA* in three mainly-
326 T (4,8) isolates (two from Azerbaijan and one from Ukraine), and the high-level resistant
327 stop codon mutation Gln58STOP which was detected in three Beijing (2,2,1) isolates from
328 Ukraine (Fig. 5A). Two DLM-resistant mutations in the *fbiA-fbiB* region were seen in a
329 single isolate, while the *fbiB* mutation Asp224Asn was found in two Delhi-CAS (3) isolates
330 from Bangladesh. Mutation type Ile208Val in *fbiA* was the most prevalent and seen in 22
331 isolates, all belonging to the Euro-American lineage (Clade1; 4,1,2) and isolated in South
332 Africa ($n= 19$), Bangladesh ($n= 2$) and Ukraine ($n= 1$) (Fig. 5B). Four of the seven DLM-
333 resistant *fbiC* mutation types were seen in single isolates, one was observed in two
334 isolates while two were more prevalent: the frameshift mutation Ala855fs (a deletion of 62
335 nt) which was detected in 30 isolates from South Africa belonging to eight different
336 lineages, and mutation Ala835Val detected in 18 EAI (1,1,3) isolates from Bangladesh only
337 (Fig. 6). Of note, most of the DLM-resistant strains with high-level of DLM MIC were
338 isolated in Bangladesh (75%) and mutations were detected in *ddn* (46%), *fbiC* (31%), *fbiA*
339 (15%) and *fbiB* (8%) (Dataset S1).

340 Cluster analysis by distance matrix was also performed in order to understand if the
341 observation of three or more isolates with mutations associated with DLM resistance were
342 potential clonal clusters. Results showed that the three Beijing (2,2,1) isolates harbouring
343 the stop codon mutation Gln58STOP in *ddn* gene were part of the same transmission
344 chain, at 12 SNPs cut-off (Fig. S2). Moreover, ten other clusters of two isolates each were

345 identified in all the other groups harbouring mutation associated with DLM resistance: four
346 clusters of isolates with the Ile208Val mutation in *fbiA*, one cluster of isolates with Ala855fs
347 variant in *fbiC*, two clusters of isolates with Pro2Gln in *ddn*, one cluster of isolates with
348 Asn91Thr and Val58Ile mutations in *ddn* and *fbiA* respectively, and two clusters of isolates
349 with *fbiC* Ala835Val (Fig. S2).

350 **4. Discussion and Conclusion**

351 Bedaquiline (BDQ) and delamanid (DLM) have expanded available treatment options and
352 improved treatment success rates for patients with pulmonary MDR-TB and XDR-TB (**44**,
353 **45**, **46**, **47**), including children with MDR-TB (**48**, **49**). The detection of resistance to BDQ
354 and DLM is critical to ensuring effective treatment and care for DR-TB patients and
355 preventing ongoing transmission. Although evidence for the validation and standardization
356 of efficient methods for MICs and the setting of breakpoints for BDQ and DLM continues to
357 expand (**22**, **34**, **50**, **51**), there is still a notable lack of suitable data on resistance-related
358 genomic variants (**52**). Moreover, phenotypic methods are too slow to provide early
359 indication of susceptibility status at the time of treatment initiation. An accurate
360 classification of SNPs according to their association with drug resistance is therefore
361 essential to allow the use of WGS to guide the composition of treatment regimens (**7**, **53**).
362 The fact that accurate databases with catalogued mutations are currently lacking for these
363 drugs, represent a serious limitation for molecular DST for BDQ and DLM.

364 To tackle this, we used a unique dataset containing 4795 WGS data of MTBc isolates from
365 different countries with either a higher burden of TB or MDR-TB (Azerbaijan, Bangladesh,
366 Belarus, South Africa, Pakistan and Ukraine) as a unique and accurate source of genetic
367 information for the characterization and validation of genomic variants potentially involved
368 in BDQ and DLM resistance. In particular, this study highlighted the role of genetic variants
369 for BDQ and DLM resistance development by combining the MICs results of MTBc isolates

370 with variants and the *in silico* analysis on available protein structures, paving the way for
371 the construction of an encyclopaedia of characterized mutations to be use for molecular
372 DST.

373 From the whole WGS dataset, we identified 61 different BDQ-related variants in *Rv0678*,
374 *atpE* promoter and *pepQ* genomic regions, out of which four were associated to BDQ-
375 resistant phenotype: two frameshift mutations in *Rv0678* associated to a high levels of
376 BDQ MIC and two non-synonymous mutations found associated to low levels of BDQ
377 resistance. To the best of our knowledge, among these four BDQ-resistant mutations, only
378 the frameshift mutation Gly6fs (del_16-17 gg) has been previously described in one BDQ-
379 resistant isolate (**54**). In agreement with the *in vitro* MIC experiments, the *in silico* structural
380 analysis on the *Rv0678* single monomer protein form (PDB 4N5B) showed that mutations
381 Met111Lys and Arg96Trp have very high Eris and MAESTRO $\Delta\Delta G$ Kcal/mol energy
382 values, indicating a strong destabilization of the protein folding. The mutations Gly87Arg
383 and Leu117Arg in *Rv0678* were previously described in BDQ-susceptible strains (**13**),
384 confirming the detected low MIC of 0.03 $\mu\text{g/ml}$ and 0.12 $\mu\text{g/ml}$, respectively. (Dataset S1).
385 The role of Leu117Arg remains unclear as another study described this mutation as
386 associated with both BDQ and also CLF resistance (**11**). However, our phenotypic and *in*
387 *silico* results suggest that Leu117Arg affects the dimerization of *Rv0678* causing a small
388 increase but not high-level value of BDQ MIC. Other mutations in *Rv0678* were described
389 at the same codon position but with a different amino acid change (**5, 8, 9, 11, 13**). The
390 *Rv0678* mutations Val20Phe, Ala84Glu and Arg90Pro were observed to be linked to
391 increase MICs for CLF and potentially associated to a BDQ-resistant phenotype, while
392 Val20Gly was associated with both BDQ and CLF resistance (**5, 9**). In this study, *Rv0678*
393 mutations Val20Ala and Ala84Val were found in BDQ-susceptible strains (MIC \leq 0.008
394 $\mu\text{g/ml}$), while Arg90Cys was associated with a BDQ MIC value of 0.12 $\mu\text{g/ml}$. Moreover, *in*

395 *silico* analysis confirmed that the Arg90Cys mutation can have a mild effect on protein
396 stability and a role in the small variation of BDQ MIC. Mutations Arg96Gln, Met146Thr and
397 Leu136Pro in *Rv0678* have been described with increased MIC values for BDQ (**8**, **11**,
398 **13**). In our dataset, strains harbouring mutations at the same codons were not consistently
399 phenotypically resistant, with mutations Arg96Trp, Met146Arg and Leu136Val respectively
400 showing MICs of 0.25 µg/ml (BDQ-resistant), 0.06 µg/ml (BDQ-susceptible) and 0.03
401 µg/ml (BDQ-susceptible). Again, *in silico* analysis results were in agreement with the
402 phenotypic data, showing that only the Arg96Trp mutation highly destabilized *Rv0678*
403 folding while the other two mutations showed a lower $\Delta\Delta G$ Kcal/mol energy values
404 (Dataset S2). Furthermore, the *in silico* MM/GBSA analysis, revealed that *Rv0678*
405 mutations Leu117Arg, Val120Met and Asp141His can affect the protein homodimerization,
406 which could explain the slight increase of BDQ MIC to 0.12 µg/ml for these MTBc strains,
407 yet still classified as BDQ-susceptible despite being close to the proposed cut-off.

408 Considering the five genomic regions associated with DLM phenotype (*ddn*, *fgd1*, *fbiA*,
409 *fbiB* and *fbiC*), the WGS analysis revealed a total of 164 unique mutations potentially
410 involved in DLM resistance. Apart from seven previously characterized mutations (**16**), all
411 the other 156 DLM-related variants have not been previously described earlier except for
412 Asn91Thr in *ddn*, for which we confirmed its role in DLM resistance (**18**). Phenotypic
413 results on a subset of available isolates showed that 32 different mutations, detected in all
414 of the considered genomic regions were associated to DLM resistance. Notably, the *in*
415 *silico* mutation structural analysis revealed that the effect of the point mutations in *Ddn* and
416 *Fgd1* were in agreement with the MIC results (Dataset S2). Indeed, the mutation Asn91Thr
417 in *Ddn* is directly involved in the binding with the co-factor F420-H₂ by disrupting the *Ddn*-
418 F420-H₂ interaction but also in destabilizing the *Ddn* folding and mutations Val61Gly and
419 Thr140Ile, which were observed in MTBc strains with MICs for DLM of 0.12 µg/ml and 0.5

420 µg/ml respectively, showed also a potential effect on Ddn protein folding and stability.
421 Furthermore, the *in silico* analysis indicates that three mutations in Ddn for which
422 corresponding phenotypic data were not available (Arg72Gln, Pro86Thr, Glu150Ala) may
423 have a significant impact on protein stability and thereby play a role in the DLM-resistant
424 phenotype.

425 In addition, the correlation analysis between mutations linked to BDQ- and DLM-resistant
426 phenotypes and metadata information corroborate with previously reported data,
427 suggesting the absence of links between BDQ or DLM resistance and strain lineage or
428 drug resistance profiles of MTBc isolates (**10, 16**). Globally, considering DST profiles of
429 BDQ- and DLM-resistant strains, 75% were fully susceptible, 6% were MDR-TB, and the
430 majority of them (68.7%) was from new TB cases. Moreover, the analysis of country-
431 lineage distribution did not reveal any significant correlation between BDQ/DLM-related
432 mutations and lineage groups or country of isolation. To complete this set of analyses, we
433 also performed a SNP-based distance matrix to evaluate the relatedness of strains
434 harbouring the most frequent DLM-resistant variants, showing that for these groups there
435 are no major transmission chains but only small clusters of two to three isolates, meaning
436 that these resistance-associated mutations can rise spontaneously and independently.
437 Conversely, none of the BDQ-resistant variants were detected in isolates groups.

438 In conclusion, our study provides novel and important evidence on the role of mutations
439 associated with BDQ- and DLM-resistant phenotypes. A concerning high prevalence of
440 genetic mutations associated with an increased MIC was detected in clinical isolates from
441 patients who have never been exposed to these drugs, supporting previous findings (**10,**
442 **16**). Also, our data showed that different non-synonymous or indel mutation at the same
443 nucleotide position can display a completely different phenotypic effect or different level of
444 resistance, thus reinforcing the need to accurately investigate the role of each individual

445 mutation. Equally important, these findings also showed the presence of 125 genetic
446 variants not associated with BDQ and DLM resistance, scattered over the full length of
447 each target gene. Therefore, considering the complexity of BDQ and DLM mechanisms of
448 resistance and the absence of fully standardized phenotypic tests, the development of
449 accurate molecular-based DST is wholly dependent on the establishment of a complete
450 database of validated mutations, a scenario which is comparable to the challenges
451 associated with molecular markers of resistance to pyrazinamide (PZA) (55). The
452 establishment of a common database combining data from MTBc isolates collected in a
453 large number of settings with the inclusion of different parameters (phenotype, genotype,
454 structure, and free energy analyses) is fundamental to improve our understanding of the
455 role of mutations in determining the BDQ/DLM susceptibility phenotype. Finally, this
456 database could be also beneficial to study genetic resistance to other drugs that could be
457 potentially sharing similar genetic basis of resistance such as clofazimine for BDQ and
458 pretomanid for DLM.

459 For these reasons, despite some limitations (not all strains were available for MIC
460 determination, absence of standardized methods and breakpoints for the interpretation of
461 BDQ and DLM phenotypes, absence of 3D structures of FbiA, FbiB, FbiC and PepQ
462 proteins for *in silico* investigation, lack of knowledge of other genomic regions potentially
463 involved in BDQ/DLM resistance), this work will be an important source of information for
464 new genome-based sequencing approaches for predicting BDQ and DLM resistance.

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695 Legends

696 **Table 1. List of *Rv0678* mutations detected in MTBc strains resistant to BDQ.** In the
697 table are reported all the information for each BDQ-resistant related mutations (MIC \geq 0.25
698 $\mu\text{g/ml}$) and for the MTBc isolate tested for BDQ susceptibility. ^a Genomic position in
699 reference H37Rv NC_000962.3 strain; ^b Amino acid (aa) change and nucleotide (nt)
700 change; ^c Minimum Inhibitory concentration (MIC) value in $\mu\text{g/ml}$. ^d Country of MTBc
701 isolate origin: Pakistan (PAK), Bangladesh (BGD). ^e TB patient treatment history: new TB
702 case “New”, or patient with previous TB history and treatment “Retreatment”. ^f Drug
703 resistance pattern of the isolates: MDR (multi drug resistant strain), FQs
704 (fluoroquinolones), INH (Isoniazid), RIF (Rifampicin), R/S (resistant/susceptible).

705 **Fig. 1. Amino acid sequence alignment of BDQ-susceptible frameshift mutations in**
706 ***Rv0678*.** The amino acid (aa) sequence of the translated proteins with Ile16fs and
707 Ala153fs frameshift mutations (associated to BDQ-susceptible MIC) were aligned with
708 *Rv0678* wild type aa sequence using ClustalX. In both cases the two insertions mutations
709 cause the addition of new aa residues without altering the frame of *Rv0678* protein and
710 functional residues of the protein.

711 **Fig. 2. Cartoon representation of *Rv0678* protein structures with mutations**
712 **associated to BDQ-resistant and to BDQ-susceptible phenotypes.** Carton
713 representation of the monomer present in the X-Ray unit cell (4NB5). Highlighted in sticks
714 are reported the mutations associated to BDQ resistance (red) and susceptible (green)
715 from BDQ MIC assay. *In silico* predicted mutations which could alter dimerization or DNA
716 binding function, and predicted associated to BDQ-susceptible phenotype are shown in

717 orange and in blue respectively. Cartoon zoomed representations of Rv0678 dimerization
718 domain and DNA binding domain are reported.

719 **Fig. 3. Lineage and country distributions of MTBc strains with variants in *Rv0678*,**
720 ***atpE* and *pepQ* genomic regions.** The graph reports all mutations found in *Rv0678*, *atpE*
721 and *pepQ* genomic regions showing their distribution among lineages and country of
722 isolation. The histograms refer to the number of strains in which mutations were observed
723 (Y axis). The colors of the histograms represent the different countries of isolation while
724 the patterns inside each bar represent the different lineages. On the X axis, the results of
725 the MIC test for available MTBc strains are also reported: red triangle are BDQ-resistant
726 strains (MIC > 0.25 µg/ml); yellow box, are BDQ low resistance level strains, (MIC = 0.25
727 µg/ml); green triangles are BDQ-susceptible strains (MIC ≤ 0.12 µg/ml).

728 **Table 2. List of *ddn*, *fgd1*, *fbiA*, *fbiB* and *fbiC* mutations detected in MTBc strains**
729 **resistant to DLM.** In the table are reported all the information for each DLM-resistant
730 related mutations (MIC ≥ 0.12) and for the MTBc isolates tested for DLM susceptibility. ^a
731 Genomic position in reference H37Rv NC_000962.3 strain. ^b Amino acid change and
732 nucleotide (nt) change; ^c minimum Inhibitory concentration (MIC) value in µg/ml. ^d Country
733 of MTBc isolate origin: Pakistan (PAK), Bangladesh (BGD), Ukraine (UKR), Azerbaijan
734 (AZE), South Africa (SA). ^e Patient treatment history; new TB case “New”, or patient with
735 previous TB history and treatment “Retreatment”. ^f Drug resistance pattern of the isolates:
736 MDR (multi drug resistant strain), FQs (fluoroquinolones), INH (Isoniazid), RIF
737 (Rifampicin), R/S (resistant/susceptible).

738 **Fig. 4. Cartoon representation Ddn and Fgd1 protein structures with mutations**
739 **found associated to DLM-resistant or associated to DLM-susceptible phenotype. A:**
740 Ddn protein bound to F420 cofactor (3R5R) with highlighted in sticks the resistant (red),
741 susceptible (green), *in silico* DLM-resistant associated mutations (orange) and *in silico*

742 predicted DLM-susceptible mutations (blue). F420 is shown in magenta licorice. A zoom
743 cartoon representation of Ddn bound to F420 is reported. The hydrogen bond network
744 Thr140-Ala82-Lys79-F420-H2 is shown in dashed yellow line and the Ala82 and Lys79
745 residues are shown in sticks. **B**: Fgd1 carton representation of holo- and homodimer-Fgd1
746 bound to F420 (3B4Y). Helices involved in protein dimerization are colored in cyan.
747 Highlighted in sticks the DLM-resistant (red), DLM-susceptible (green), *in silico* predicted
748 DLM-resistant mutations (orange) and *in silico* predicted DLM-susceptible mutations
749 (blue).

750 **Fig. 5. Lineage and country distributions of MTBc strains with variants in *ddn* and**
751 ***fgd1* genomic regions and *fbiA-fbiB* operon region.** The graph reports all mutations
752 found in *ddn*, *fgd1* (**A**) and *fbiA*, *fbiB* (**B**) genomic regions, showing their distribution among
753 lineages and country of isolation. The histograms refer to the number of strains in which
754 mutations were observed (Y axis). The colors of the histograms represent the different
755 countries of isolation while the patterns inside each bar represent the different lineages.
756 On the X axis, the results of the MIC test for available MTBc strains are also reported: red
757 triangle are DLM-resistant strains (MIC \geq 0.12 $\mu\text{g/ml}$); yellow box are low resistance level
758 (MIC = 0.12 $\mu\text{g/ml}$) and green triangles are DLM-susceptible strains (MIC < 0.12 $\mu\text{g/ml}$). If
759 a mutation was previously described in literature it was also reported (“S” for strain
760 susceptible to DLM or “R” for strain resistant to DLM).

761 **Fig. 6. Lineage and country distributions of MTBc strains with variants in *fbiC***
762 **genomic region.** In this graph are report all mutations found in *fbiC* genomic region
763 showing their distribution among lineages and countries of isolation. See Fig. 5 legend for
764 details.

765 **Table S1. *M. tuberculosis* genomic regions considered for bedaquiline and**
766 **delamanid resistances.**

767 ^a For promoter regions, it was considered the upstream region up to the -100 position
768 before the first nucleotide of each gene.

769 ^b The genomic positions are based on the reference genome of *M. tuberculosis* H37Rv
770 NC_000962.3.

771 **Fig. S1. Study design, number of selected *M. tuberculosis* (MTBc) isolates and DST**
772 **profile categorization. A:** phenotypic drug susceptibility test (pDST) of the whole
773 collection which classified MTBc isolates in not-MDR, MDR and XDR strains. **B:** Flow chart
774 scheme of isolates selection and stratifications. The blue scheme refers to MTBc isolates
775 selected for DLM-related mutations while the red once to MTBc isolates selected for BDQ-
776 related mutations. ^a The WHO approved study list includes 4795 whole genome
777 sequencing (WGS) samples (accession number SRP128089). ^b Stratification by the
778 phenotypic resistance profile for the selected isolates. Abbreviations: **not-MDR**, fully
779 susceptible strains (full-S), mono resistant to rifampicin (RIF) or to isoniazid (INH); **MDR**,
780 multidrug resistant strains, resistant almost to INH and RIF; **INH-R**, resistant to isoniazid;
781 **RIF-R**, resistant to rifampicin; **FQ-R**, resistant to fluoroquinolones; **Fully-S**: susceptible to
782 INH and RIF; **Pre-XDR**, MDR resistant also to FQ or second line injectables; **XDR**, MDR
783 plus resistance to FQs and second-line injectables.

784 **Dataset S1. Samples general database.**

785 General database with all information of selected MTBc strains harbouring at least one
786 mutation pattern in candidate genes for BDQ and DLM resistance. The excel database is
787 divided in two sheets named “mutations list for BDQ” and “mutations list for DLM”. ^a WGS
788 analysis (genomic regions for BDQ/DLM resistance) in which are report the list of
789 mutations from WGS analysis with information of genomic locus, gene ID, genomic
790 coordinate (reference strain H37Rv NC_000962.3), amino acid substitution and type of
791 mutation. ^b MIC value results (µg/mL) from REMA assay (DLM/BDQ) of selected isolates

792 (for several mutations two different isolates were tested); empty cell means that the strain
793 with that specific mutation was not available. ^c Previously reported mutations: If a mutation
794 was previously reported it is indicated if linked or not to resistance phenotype. ^e
795 Information on selected samples (isolates 1 and 2): WGS sample name in the WHO
796 database, country of origin, lineage (coll. lineage) and DST profile of MTBc isolates
797 selected for MIC test. ^d Lineage/Country mutation frequency distribution: numbers of MTBc
798 isolates carrying mutations among countries of origin and strain lineage.

799 **Dataset S2. Mutation structural analysis.**

800 General database with all results from the *in silico* analysis of point mutations for the
801 available crystal structures of proteins: Ddn (PDB 3R5R), Fgd1 (PDB 3B4Y) and Rv0678
802 (PDB 4NB5). Free energy calculation ($\Delta\Delta G$ kcal/mol) of all amino acid change mutations
803 were performed with Eris, an automated estimator of protein stability and MAESTRO, an
804 approach for multi agent stability prediction upon point mutations. DrugScore (DSX)
805 software, a Knowledge-Based Scoring Function for the Assessment of Protein–Ligand
806 Complexes, was used for Ddn-F420 and Fgd1-F420 complexes analysis. The calculation
807 of mutations effect on Rv0678 dimer stability was performed using MMPBSA.py program
808 within Amber14 suite. See materials and methods for details.

809 **Figure S2. Heatmap of SNP based cluster analysis by distance matrix.** The figure
810 shows the SNP-based cluster analysis results of six MTBc strains groups harbouring the
811 most frequent DLM-resistant related mutations. Colour scale in the square refers to the
812 number of SNPs between each strain (12 SNPs threshold is reported from white to blue).
813 The max number of SNPs are set to 15. MTBc lineages information is also reported.

814 **Text S1. Supplementary material and methods.** Supplementary information about
815 protocols used for REMA and *in silico* analyses.

816

817 **Tables:**

818 **Table 1.**

<i>Gene locus</i>	<i>Genomic coordinate</i> ^a	<i>Mutation (SNPs and Indels)</i> ^b	<i>BDQ MIC (µg/mL)</i> ^c	<i>Country</i> ^d	<i>Lineage (coll. lineage)</i>	<i>Treatment history</i> ^e	<i>DR pattern</i> ^f
<i>Rv0678</i>	779005	Gly6fs (Del_16-17 gg)	0.5	PAK	Delhi-CAS (3)	New	MDR, FQ-R
<i>Rv0678-Rv0678</i>	779016 779263	Gln9fs (Ins_27 c) Tyr92fs (Ins_274 a)	0.5	PAK	EAI (1,1,2)	Retreatment	MDR
<i>Rv0678</i>	779275	Arg96Trp (cgg/Tgg)	0.25	BGD	Delhi-CAS (3)	Retreatment	RIF, INH S
<i>Rv0678</i>	779321	Met111Lys (tag/aAg)	0.25	BGD	Haarlem (4,1,2,1)	New	RIF, INH S

819

820 **Table 2.**

<i>Gene locus</i>	<i>Genomic coordinate</i> ^a	<i>Mutation (SNPs and Indels)</i> ^b	<i>DLM MIC (µg/mL)</i> ^c	<i>Country</i> ^d	<i>Lineage (coll. Lineage)</i>	<i>Treatment history</i> ^e	<i>DR pattern</i> ^f
<i>ddn</i>	3986846	Met1fs (Del_2 t)	2	BGD	Eur-Amer.(4,1,2)	New	RIF, INH S
<i>ddn</i>	3986848	Pro2Gln (ccg/cAg)	0.25	AZE	mainly-T (4,8)	New	RIF, INH S
<i>ddn</i>	3986885	Ser14fs (Del_41 g)	> 4	BGD	Beijing (2,2,1)	Retreatment	MDR
<i>ddn-fbiA</i>	3986923 3640445	Trp27Stop (tgg/tAg) prom (g-18a)	> 4	BGD	EAI (1,1,3)	Retreatment	MDR
<i>ddn</i>	3986932	Arg30His (cgc/cAc)	1	BGD	EAI (1,1,3)	Retreatment	RIF, INH S
<i>ddn</i>	3986944	Gly34Glu (ggg/gAg)	0.12	AZE	LAM (4,3,3)	New	RIF, INH S
<i>ddn</i>	3987015	Gln58Stop (cag/Tag)	> 4	UKR	Beijing (2,2,1)	Retreatment	MDR, FQ-R
<i>ddn-fbiA</i>	3987025 3641164	Val61Gly (gtc/gGc) Ile208Val (atc/Gtc)	0.12	SA	Eur-Amer.(4,1,2)	New	RIF, INH S
<i>ddn-fbiA</i>	3987115 3640714	Asn91Thr (aac/aCc) Val58Ile (gtc/Atc)	0.25	AZE	mainly-T (4,8)	New	RIF, INH S
<i>ddn</i>	3987262	Thr140Ile (acc/aTc)	0.5	BGD	S-type (4,4,1,1)	New	RIF, INH S
<i>fgd1</i>	491723	Gly314Glu (gga/gAa)	0.12	BGD	Beijing (2,2,1)	New	RIF, INH S
<i>fbiA</i>	3640546	Lys2Glu (aag/Gag)	0.12	AZE	Beijing (2,2,1)	Retreatment	RIF, INH S
<i>fbiA</i>	3641002	Val154Ile (gta/Ata)	0.12	BGD	Beijing (2,2,1)	Retreatment	MDR, FQ-R
<i>fbiA-fbiB</i>	3641018 3642877	Pro159Gln (ccg/cAg) Lys448Arg (aag/aGg)	0.12	BGD	Delhi-CAS (3)	Retreatment	RIF, INH S
<i>fbiA</i>	3641164	Ile208Val (atc/Gtc)	0.25	BGD	Eur-Amer.(4,1,2)	New	RIF, INH S
<i>fbiA</i>	3641167	Ile209Val (atc/Gtc)	0.5	BGD	Eur-Amer.(4,5)	New	RIF, INH S
<i>fbiA</i>	3641403	Cys287Stop (tgc/tgA)	4	PAK	EAI (1,1,2)	New	RIF, INH S
<i>fbiA</i>	3641453	Arg304Gln (cgg/cAg)	0.25	PAK	Delhi-CAS (3)	New	MDR
<i>fbiB</i>	3642195	Gly221Ser (ggc/Agc)	0.12	BGD	Beijing (2,2,2)	New	FQ-R
<i>fbiB</i>	3642204	Asp224Asn (gac/Aac)	0.5	BGD	Delhi-CAS (3)	New	RIF, INH S
<i>fbiB</i>	3642351	Gly273Arg (ggc/Cgc)	0.25	BGD	X-Type (4,1,1,3)	New	RIF, INH S

fbiC	1303241	Tyr104Cys (tat/tGt)	0.5	AZE	Beijing (2,2,1)	Retreatment	RIF, INH S
fbiC	1303265	Gly112Ala (ggc/gCc)	0.12	BGD	EAI (1,1,3)	New	RIF, INH S
fbiC	1303612	Leu228Phe (ctc/Ttc)	0.12	BGD	EAI (1,1,3)	New	RIF, INH S
fbiC- fbiB	1303769 3642223	Ser280Leu (tcg/tTg) Arg230Gln (cgg/cAg)	0.12	BGD	EAI (1,1,3)	New	RIF, INH S
fbiC	1304498	Pro523Leu (cct/cTt)	0.5	BGD	EAI (1,1,3)	New	RIF, INH S
fbiC	1305101	Asn724Ser (aac/aGc)	0.25	BGD	Beijing (2,2,1)	New	RIF, INH S
fbiC	1305215	Ser762Asn (agc/aAc)	0.12	BGD	Delhi-CAS (3)	Retreatment	RIF, INH S; FQ-R
fbiC	1305434	Ala835Val (gcg/gTg)	0.5	BGD	EAI (1,1,3)	New	INH R
fbiC	1305494	Ala855fs (Del 62 nt)	0.5	SA	Haarlem (4,1,2,1)	New	RIF, INH S
fbiC- fbiB	1305494 3642874	Ala855fs (Del 62 nt) Leu447Arg (ctg/cGg)	0.25	SA	mainly-T (4,8)	Retreatment	MDR
fbiC	1305496	Ala856Pro (gcc/Ccc)	0.25	BGD	EAI (1,1,3)	New	RIF, INH S

821

822

823

824 **Table S1**

	<i>locus name</i> ^a	<i>genomic region</i> ^b
Delamanid (DLM)	<i>fbiA_ups</i>	[3640142-3640542]
	<i>fbiA (Rv3261)</i>	[3640543-3641538]
	<i>fbiB (Rv3262)</i>	[3641535-3642881]
	<i>fbiC_ups</i>	[1302682-1302930]
	<i>fbiC (Rv1173)</i>	[1302931-1305501]
	<i>fgd1_ups</i>	[490683-490782]
	<i>fgd1 (Rv0407)</i>	[490783-491793]
	<i>ddn_ups</i>	[3986744-3986843]
	<i>ddn (Rv3547)</i>	[3986844-3987299]
Bedaquiline (BDQ)	<i>atpE_ups</i>	[1460997-1461044]
	<i>atpE (Rv1305)</i>	[1461045-1461290]
	<i>Rv0678_ups</i>	[778890-778989]
	<i>Rv0678</i>	[778990-779487]
	<i>pepQ_ups</i>	[2860419-2860518]
	<i>pepQ (Rv2535)</i>	[2859300-2860418]

825

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*****
Rv0678      VSVNDGVDQMGAEPDIMEF----VEQMGGYFESRSLTRLAGRLLGWLLVCDPEROSSEELATALAASSGGISTNARMLIQ 76
Rv0678_Ile16fs VSVNDGVDQMGAEPDIMEFVMEFVEQMGGYFESRSLTRLAGRLLGWLLVCDPEROSSEELATALAASSGGISTNARMLIQ 80
Rv0678_Alal53fs VSVNDGVDQMGAEPDIMEF----VEQMGGYFESRSLTRLAGRLLGWLLVCDPEROSSEELATALAASSGGISTNARMLIQ 76
1.....10.....20.....30.....40.....50.....60.....70.....80

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***** *
Rv0678      FGFIERLAVAGDRRTYFRLRPNAFAAGERERIRAMAEQLDLADVGLRALGDAPPQRSRRLREMRDLLAYMENVVSDALG- 155
Rv0678_Ile16fs FGFIERLAVAGDRRTYFRLRPNAFAAGERERIRAMAEQLDLADVGLRALGDAPPQRSRRLREMRDLLAYMENVVSDALG- 159
Rv0678_Alal53fs FGFIERLAVAGDRRTYFRLRPNAFAAGERERIRAMAEQLDLADVGLRALGDAPPQRSRRLREMRDLLAYMENVVSDRPGA 156
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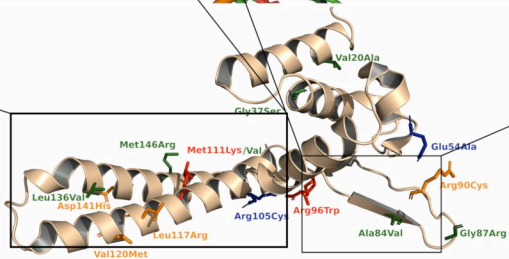
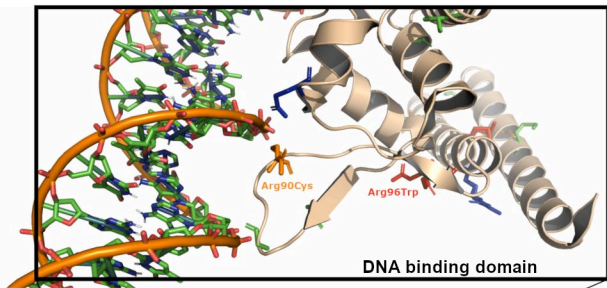
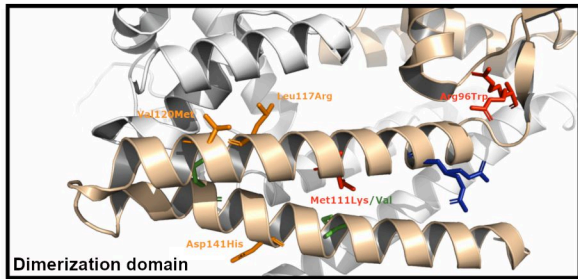


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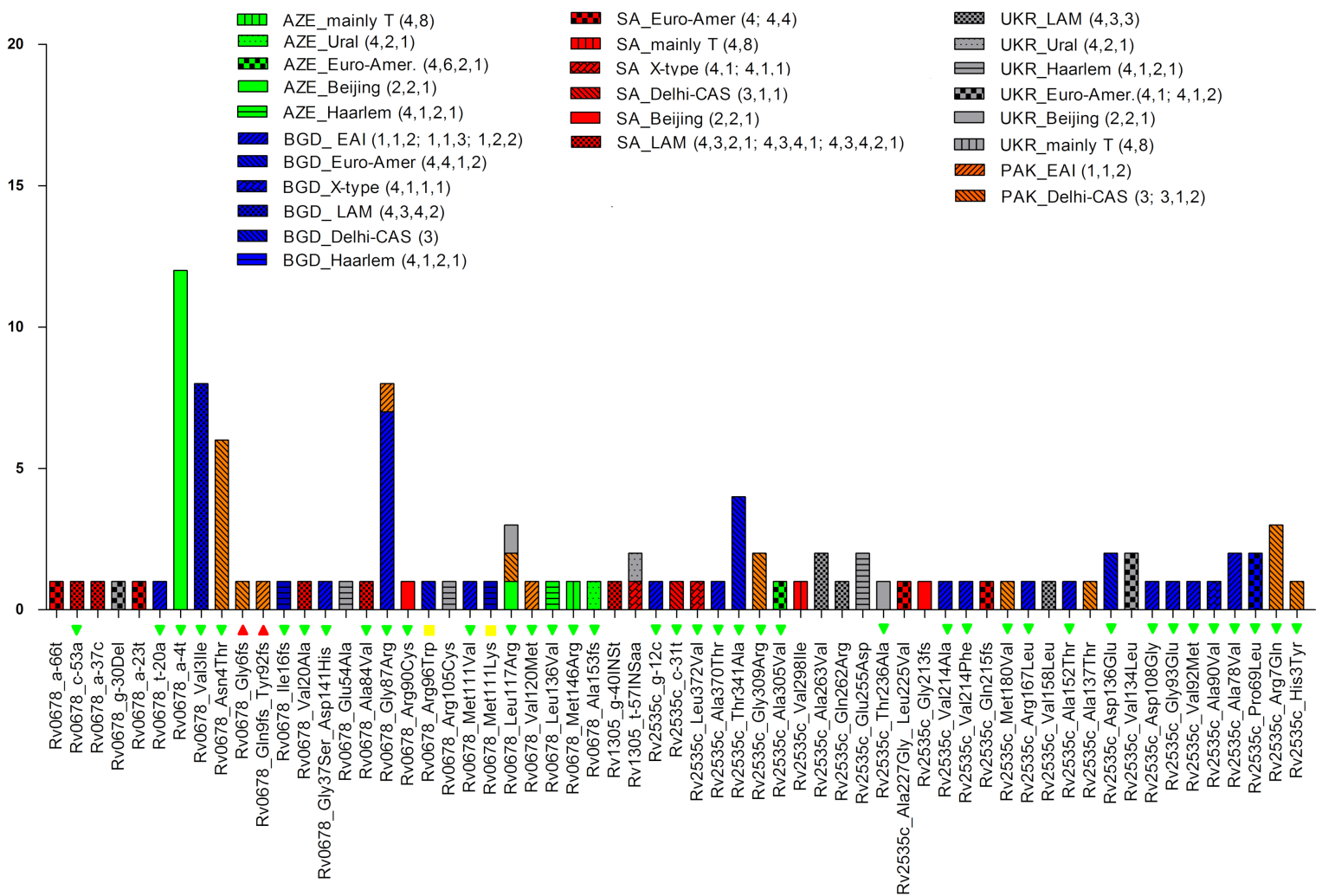
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Rv0678_Alal53fs IQPANRRGRLLMSNLAI 172
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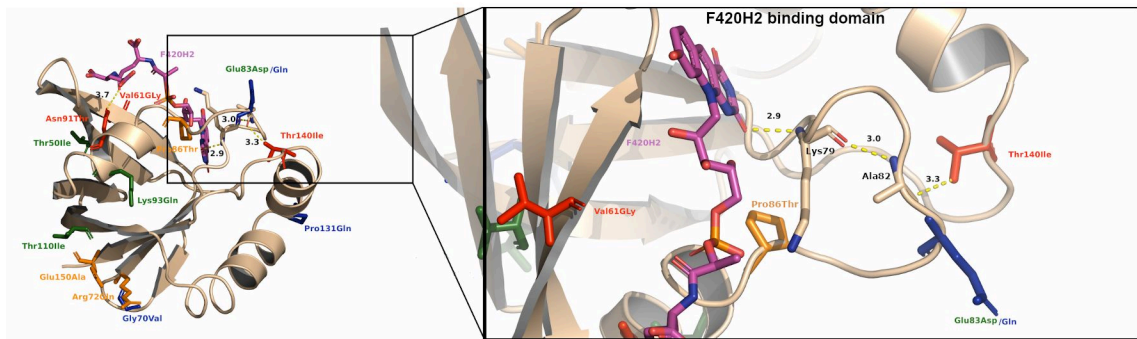




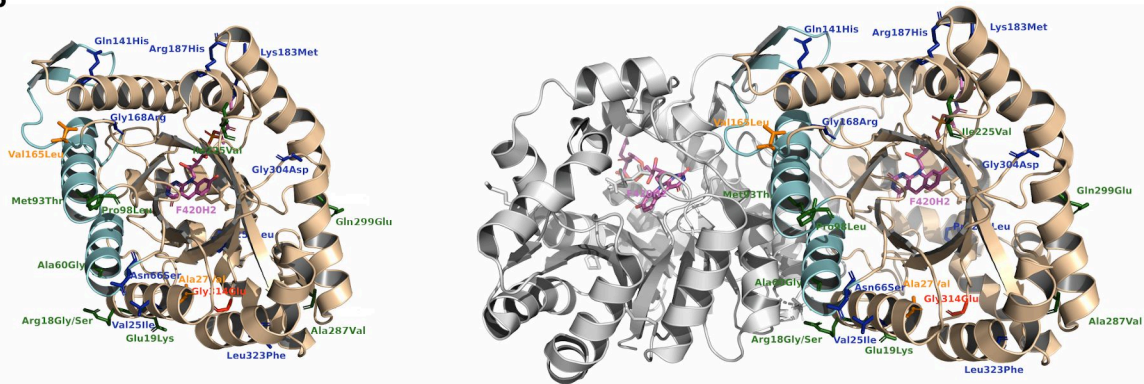
N. of MTBc isolates

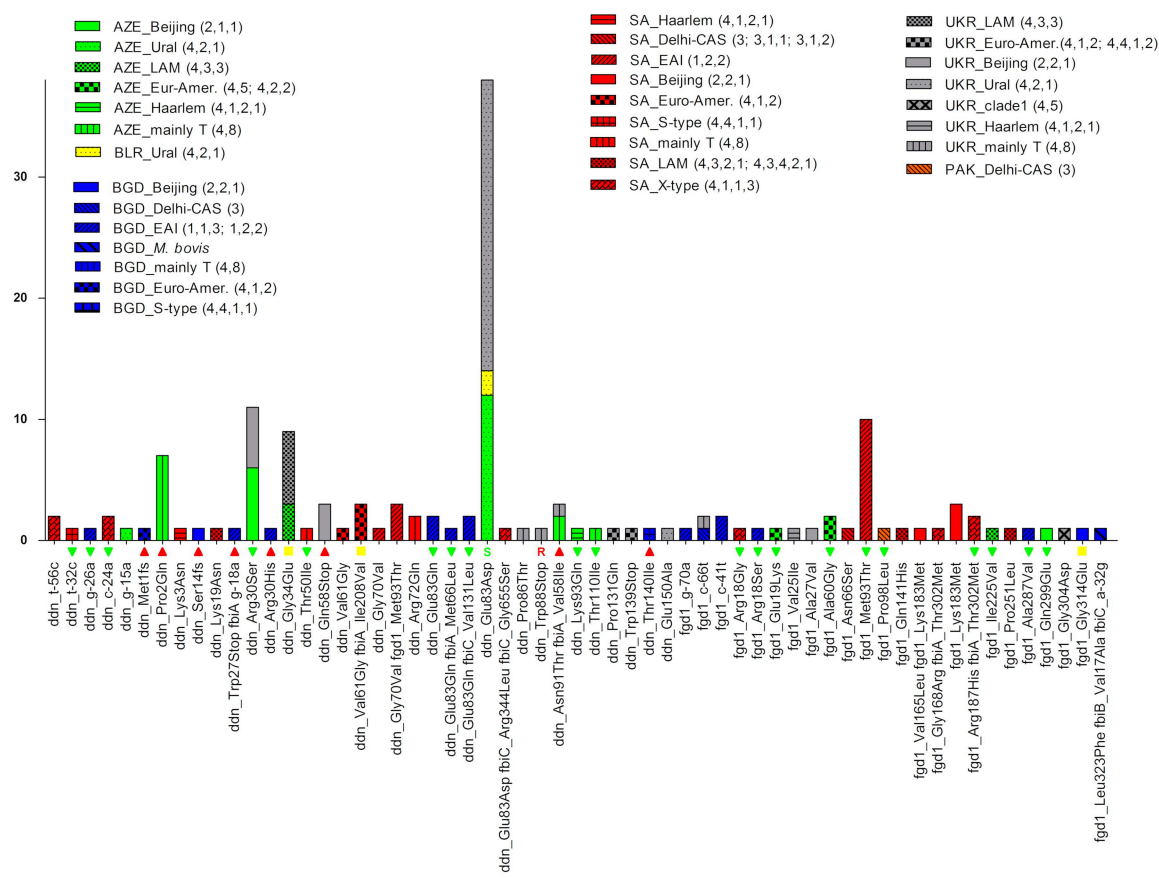
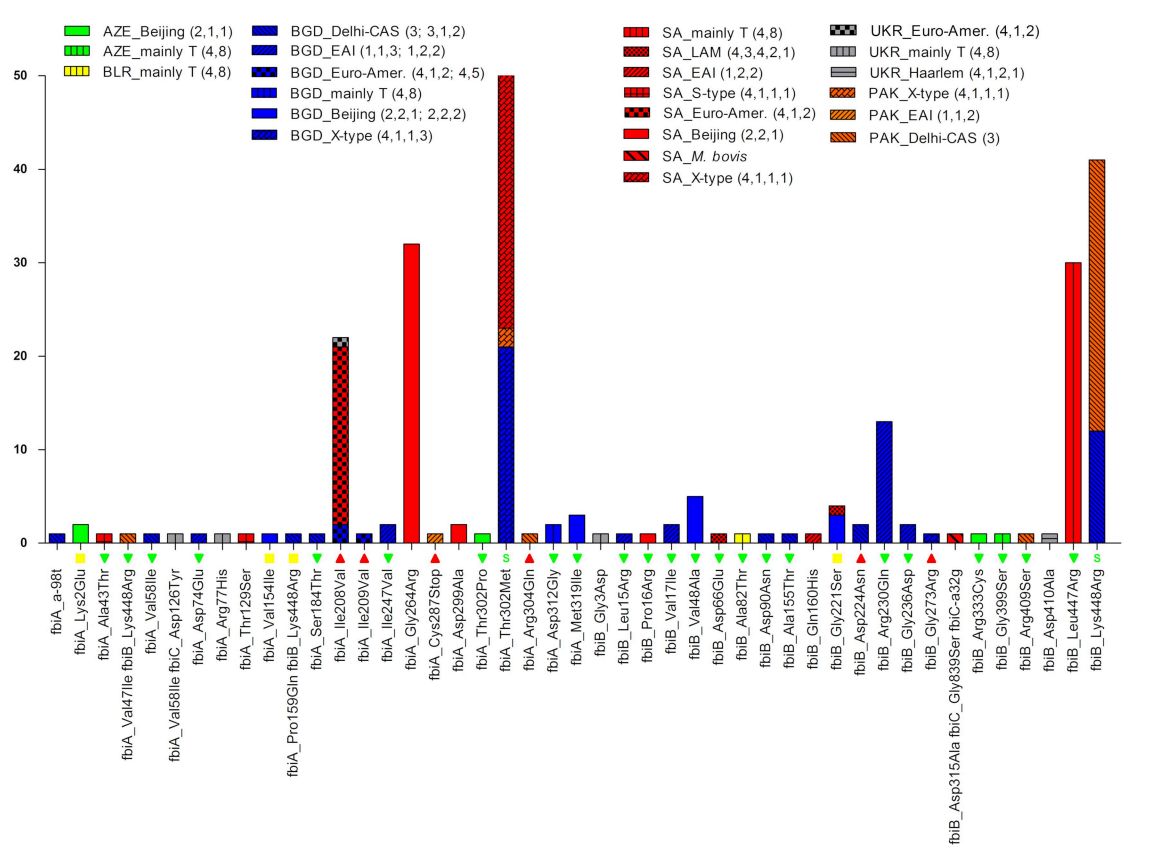


A



B



A**N. of MTBc isolates****B****N. of MTBc isolates**

N. of MTBc isolates

