Whole Genome Sequencing Identifies Novel Mutations Associated With Bedaquiline Resistance in Mycobacterium tuberculosis

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Bedaquiline (BDQ), a new antitubercular agent, has been used to treat drug-resistant tuberculosis (TB). Although mutations in atpE, rv0678, and pepQ confer major resistance to BDQ, the mechanisms of resistance to BDQ in vitro and in clinical settings have not been fully elucidated. We selected BDQ-resistant mutants from 7H10 agar plates containing 0.5 mg/L BDQ (the critical concentration) and identified mutations associated with BDQ resistance through whole genome sequencing and Sanger sequencing. A total of 1,025 mutants were resistant to BDQ. We randomly selected 168 mutants for further analysis and discovered that 157/168 BDQ-resistant mutants harbored mutations in rv0678, which encodes a transcriptional regulator that represses the expression of the efflux pump, MmpS5–MmpL5. Moreover, we found two mutations with high frequency in rv0678 at nucleotide positions 286–287 (CG286–287 insertion; accounting for 26.8% [45/168]) and 198–199 (G198, G199 insertion, and G198 deletion; accounting for 14.3% [24/168]). The other mutations were dispersed covering the entire rv0678 gene. Moreover, we found that one new gene, glpK, harbors a G572 insertion; this mutation has a high prevalence (85.7%; 144/168) in the isolated mutants, and the minimum inhibitory concentration (MIC) assay demonstrated that it is closely associated with BDQ resistance. In summary, we characterized 168/1,025 mutants resistant to BDQ and found that mutations in rv0678 confer the primary mechanism of BDQ resistance. Moreover, we identified a new gene (glpK) involved in BDQ resistance. Our study offers new insights and valuable information that will contribute to rapid identification of BDQ-resistant isolates in clinical settings.

Keywords: Mycobacterium tuberculosis, bedaquiline, drug resistance, rv0678, glpK
INTRODUCTION

Multidrug-resistant tuberculosis (MDR-TB), caused by drug-resistant Mycobacterium tuberculosis (Mtb), results from various factors, including substandard treatment regimens, treatment nonadherence, drug malabsorption, and drug–drug interactions during anti-TB therapy. This hampers the efforts of the World Health Organization (WHO)’s End TB Strategy (Nguyen et al., 2018; World Health Organization, 2019). In 2018, MDR-TB including rifampicin-resistant TB accounted for 3.4% of new TB cases and 18% of previously treated cases (World Health Organization, 2019). Moreover, approximately 6.2% of MDR-TB cases develop extensively drug-resistant TB (World Health Organization, 2019). Therefore, there is an urgent need to discover new and effective anti-TB drugs.

Bedaquiline (BDQ), a new antitubercular agent, has better efficacy against both drug-susceptible and drug-resistant Mtb in vitro (Andries et al., 2005) and improved treatment outcomes and cure rates among patients with drug-resistant TB (Diacon et al., 2014; Schnippel et al., 2018). As a result, WHO recommended the addition of BDQ to treatment regimens for drug-resistant TB (WHO, 2019). However, since the introduction of BDQ to treatment regimens, BDQ resistance has also emerged (Veziris et al., 2017).

BDQ exerts its antitubercular activity by inhibiting the activity of the F$_{1}$F$_{0}$-adenosine triphosphate (ATP) synthase encoded by the atpE gene (Andries et al., 2005). Therefore, mutations in atpE (A28V, A28P, G61A, A63P, and I66M) are associated with high levels of BDQ resistance (10–128-fold MIC). BDQ-resistant strains are currently being selected in vitro studies, but they are rarely found in patients with TB (Nieto Ramirez et al., 2020), thus suggesting that mutations in the atpE gene have a fitness cost within these patients in comparison to in vitro cultures. Moreover, there is another ATP-synthase-independent mechanism of BDQ resistance (Nguyen et al., 2018). The drug efflux system functions as a non-target-based mechanism related to resistance against many antimicrobials, including BDQ (Andries et al., 2014). The rv0678 gene encodes the Mtb transcriptional repressor of the MmpS5–MmpL5 efflux system, and high frequency mutations in the rv0678 gene result in low BDQ resistance (2–8-fold MIC) in vitro and in clinical settings (Hartkoorn et al., 2014; Coeck et al., 2015). Mutations in the rv0678 gene result in damaged function and upregulation of the MmpS5–MmpL5 efflux system. Interestingly, the MmpS5–MmpL5 efflux pump-based mechanism also exists in clofazimine resistance (Hartkoorn et al., 2014).

A previous study showed that pepQ gene mutations also lead to low-level BDQ resistance (up to fourfold) in mice (Almeida et al., 2016; Degiacomi et al., 2020) To our knowledge, mutations in atpE, rv0678, and pepQ gene have shown to be the major mechanisms of Mtb resistant to BDQ. However, Mtb isolates from one patient showed an increased MIC to BDQ, but no mutations were found in atpE, Rv0678, and pepQ and their respective upstream regions (Andres et al., 2020), suggesting the existence of other undetermined mechanisms of BDQ resistance. Consistent with this, Peterson et al. found that BDQ-treated Mtb could initiate a regulatory network regulated by Rv0324 and Rv0880 that coordinates multiple resistance mechanisms to push Mtb into a BDQ-tolerant state (Peterson et al., 2016). Therefore, the identification of new BDQ resistance-related genes will contribute to a comprehensive understanding of BDQ-resistant mechanisms that are critical for reducing the emergence of resistance and anti-TB drug discovery.

This study investigated BDQ-resistance-related mutations through Sanger and whole genome sequencing (WGS).

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The Mtb H37Rv strain was cultured in Middlebrook 7H9 broth (7H9; Difco, Detroit) liquid medium containing 0.05% Tween 80 and 10% oleic acid, albumin, dextrose, and catalase (OADC) or plated on Middlebrook 7H10 agar medium (Difco, Detroit) containing 10% OADC and 0.5% glycerol.

BDQ-Resistant Mutant Selection

BDQ-resistant mutants were screened, as previously shown (Zhang et al., 2015; Pi et al., 2019). In brief, log phase Mtb H37Rv cultures were spread on 7H10 agar plates inoculated with 0.5 mg/L BDQ (Abcam, Cambridge, UK) and incubated at 37°C for 4 weeks. The critical concentration, which is approximately eightfold MIC, was chosen based on the MIC values reported in previous studies: clinical BDQ-resistant isolates have MIC values >0.25 mg/L (Veziris et al., 2017), and low-level BDQ resistant strains (with 2–8-fold MIC) are often seen both in vitro and in clinical settings; therefore, 0.5 mg/L was used for mutant selection. All colonies were picked and transferred onto new Middlebrook 7H10 agar plates containing 0.5 mg/L BDQ to demonstrate their resistance.

Gene Mutation Analysis Through WGS

Genomic DNA extracted from BDQ-resistant strains and Mtb H37Rv using cetyltrimethylammonium bromide (Benjak et al., 2015) was analyzed by WGS using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) (Zhang et al., 2015). For each mutant, approximately 500 Mb to 1.5 Gb (110- to 350-fold genome coverage) sequences were produced, followed by the removal of the barcodes. Single-nucleotide variants (SNVs), insertions, and deletions harboring 1–58 bp were sorted and called with more than four reads using the Mtb H37Rv genome (NC_000962.3) as a reference. The proline–glutamic acid/proline–glutamic acid proline–glutamic acid family genes with mutations were not included in the analysis. Mutations harbored by MtbH37Rv contrasting to the online genome (NC_000962.3) were also removed and not analyzed.

Abbreviations: ATP, adenosine triphosphate; BDQ, Bedaquiline; HT, homopolymeric tract; MDR-TB, multidrug-resistant tuberculosis; MIC, minimum inhibitory concentration; Mtb, Mycobacterium tuberculosis; OADC, oleic acid, albumin, dextrose, and catalase; TB, tuberculosis; WGS, whole genome sequencing; WT, wild type.
PCR and Sanger Sequencing

The BDQ-resistance-related genes were subjected to PCR amplification using the primers listed in Supplementary Table S1 and the genomic DNA of BDQ-resistant isolates selected in vitro as a template. The obtained PCR products were sent for sequencing using the Sanger method to determine the mutations harbored in these genes in the isolated BDQ-resistant mutants.

Antimicrobial Susceptibility Testing

The indicated mutants were picked from 7H10 agar plates in a tube containing 2 ml 7H9 liquid medium containing 10% OADC. After dispersing with a BACspreader (TB Healthcare, Guangdong, China) for 1 min, the bacteria suspension was prepared. BDQ was double diluted in 7H9 liquid medium containing 10% OADC (20–0.078 mg/L). One hundred microliters of each dilution and 100 µl of the suspension were then mixed in a 96-well plate. MICs were shown as the lowest concentration of BDQ at which no visible growth was observed.

Data Availability

The WGS data were submitted to the Sequence Read Archive of the National Center for Biotechnology Information (PRJNA766993).

RESULTS

Selection of BDQ-Resistant Isolates

In order to select mutants resistant to BDQ, approximately 5×10⁹ bacteria were spread onto 7H10 agar plates containing 0.5 mg/L BDQ as numeredated by the colony-forming units count in the absence of antibiotics. A total of 1.025 BDQ-resistant isolates were selected, and 168 mutants were randomly selected from the plates and sent for WGS (101 mutants) and PCR (67 mutants) analysis. The mutation frequency of BDQ-resistant mutants was approximately 2×10⁻⁷.

WGS Identification of BDQ Resistance-Related Mutations

To determine the possible mechanisms of resistance to BDQ, the BDQ-resistant mutants and the parent strain H37Rv were performed WGS and genome sequence comparisons. As shown in Table 1, most of the BDQ-resistant mutants (94/101) had rv0678 gene mutations, including SNVs, deletions, insertions, and deletion and insertion. Insertion and deletion mutations in the rv0678 gene cause a frameshift and may result in the loss of its function. No BDQ resistance mutations were identified in the atpE and pepQ genes in these mutants (Table 1). Given that the WGS results suggested that mutations in rv0678 conferred the primary resistance mechanism against BDQ for the mutants identified, PCR and Sanger sequencing of the rv0678 gene were performed on a different set of 67 mutants (Table 2). Similar results were observed, and 63/67 mutants with rv0678 gene mutations (including SNVs, deletion, and insertion) were identified (Table 2). In accordance with its function, which acts as a repressor of the MmpS5-MmpL5 efflux pump, these results reveal that the rv0678 mutation leads to primary resistance to BDQ.

Among all the mutants subjected to WGS and Sanger sequencing analysis, we found two high frequency mutations in the rv0678 gene. One hotspot was found at nucleotide positions 286–287 (CG286–287 insertion) and accounted for 26.8% (45/168) of the mutants (Tables 1, 2). The second hotspot was found at nucleotide positions 198–199 (G198 deletion and insertion and G199 insertion) and accounted for 14.3% (24/168) of the mutants (Tables 1, 2). The other mutations were widespread throughout the entire rv0678 gene with no apparent clustering (Tables 1, 2).

In addition, we identified a few new, previously undetermined genes that may be related to BDQ resistance. Those newly identified genes include glpK, cobQ2, pitA, gid, rv2426c, rv2820c, rv3510c, rv0071, cya, rv2477c, lppB, rv0953c, rv2823c, rv4045, fhaA, rv2722, trecR, rv3785, and rv1723 (Table 3). Moreover, most rv0678 mutants also harbored mutations in the newly identified genes, regardless of the rv0678 mutation. In particular, we found highly prevalent mutations in glpK in the selected isolates, in addition to rv0678 mutations (Table 3). Furthermore, our results showed that most BDQ-resistant mutants (86/101) with mutations in rv0678 also harbored the G572 insertion mutation in glpK (Table 3). Interestingly, a proportion of BDQ-resistant mutants (6/101) with wild-type (WT) rv0678 had a G572 insertion mutation in glpK (Table 3). PCR and Sanger sequencing analysis performed on the remaining 67 BDQ-resistant mutants showed similar results (58/67 mutant harboring rv0678 mutations and the G572 insertion mutation in glpK; 3/67 harboring WT rv0678 and G572 insertion mutation in glpK; Table 2). Therefore, it is likely that insertion mutations in the glpK gene are associated with BDQ resistance. To determine the relationship between the glpK gene mutation and BDQ resistance, an MIC assay was performed. rv0678 had a G572 insertion mutation in glpK; 3/67 harboring WT rv0678 and G572 insertion mutation in glpK (Table 2). Moreover, mutants harboring the WT glpK gene, rv0678 (GC285–286 insertion), and rv2820c (A342T) mutants had the same MIC as that of mutants with WT rv0678, glpK (G572 insertion), and rv2820c (A342T) gene mutations (Table 4). Interestingly, a proportion of BDQ-resistant mutants (6/101) with mutations in rv0678 also harbored the G572 insertion mutation in glpK (Table 3). Interestingly, a proportion of BDQ-resistant mutants (6/101) with wild-type (WT) rv0678 had a G572 insertion mutation in glpK (Table 3). PCR and Sanger sequencing analysis performed on the remaining 67 BDQ-resistant mutants showed similar results (58/67 mutant harboring rv0678 mutations and the G572 insertion mutation in glpK; 3/67 harboring WT rv0678 and G572 insertion mutation in glpK; Table 2). Therefore, it is likely that insertion mutations in the glpK gene are associated with BDQ resistance. To determine the relationship between the glpK gene mutation and BDQ resistance, an MIC assay was performed. rv0678 had a G572 insertion mutation in glpK; 3/67 harboring WT rv0678 and G572 insertion mutation in glpK (Table 2). Moreover, mutants harboring the WT glpK gene, rv0678 (GC285–286 insertion), and rv2820c (A342T) mutants had the same MIC as that of mutants with WT rv0678, glpK (G572 insertion), and rv2820c (A342T) gene mutations (Table 4). Together, these results suggest that insertion mutations in glpK may be responsible for resistance to BDQ.

DISCUSSION

In the present study, to better characterize the mechanisms of BDQ resistance, we identified 1,025 BDQ-resistant mutants from approximately 5×10⁹ bacteria. The mutation frequency of BDQ-resistant mutants was approximately 2×10⁻⁷, which is consistent with the frequency observed in previous studies (Andries et al., 2005; Huitric et al., 2010; Nguyen et al., 2018). Our results indicate that rv0678 mutations may have a low cost in vitro and in clinical settings without causing any loss in fitness (Nguyen et al., 2018), which might account for the high rate of BDQ
Table 1: Mutation Analysis of 101 BDQ-Resistant Mutants of Mtb by Whole-Genome Sequence Analysis

| Gene  | Mutation type | Nucleotide change                  | Amino acid change | Mutant count |
|-------|---------------|-----------------------------------|-------------------|--------------|
| rv0678 | nonsynonymous SNV | T124G                             | W42G              | 1            |
| rv0678 | nonsynonymous SNV | T78G                              | Y26stop           | 1            |
| rv0678 | nonsynonymous SNV | T31C                              | L44P              | 1            |
| rv0678 | nonsynonymous SNV | T425G                             | L142R             | 8            |
| rv0678 | nonsynonymous SNV | G120T                             | L40F              | 1            |
| rv0678 | nonsynonymous SNV | G308C                             | G103A             | 1            |
| rv0678 | nonsynonymous SNV | C214T                             | R72W              | 2            |
| rv0678 | nonsynonymous SNV | T254A                             | V85D              | 1            |
| rv0678 | nonsynonymous SNV | T179C                             | L60P              | 5            |
| rv0678 | nonsynonymous SNV | G71T                              | G24V              | 1            |
| rv0678 | nonsynonymous SNV | T341C                             | G103A             | 1            |
| rv0678 | deletion       | G184 deletion                      | 62 codon shift    | 1            |
| rv0678 | deletion       | A415 deletion                      | 157 codon shift   | 1            |
| rv0678 | deletion       | T451 deletion                      | 73 codon shift    | 2            |
| rv0678 | deletion       | G19 deletion                       | 67 codon shift    | 5            |
| rv0678 | deletion       | G216 deletion                      | 117 codon shift   | 1            |
| rv0678 | deletion       | C158 deletion                      | 53 codon shift    | 1            |
| rv0678 | deletion       | T95 deletion                       | 142 codon shift   | 1            |
| rv0678 | deletion       | A415 deletion                      | 139 codon shift   | 1            |
| rv0678 | deletion       | G19 deletion                       | 7 codon shift     | 1            |
| rv0678 | deletion       | G19 insertion                      | 67 codon shift    | 10           |
| rv0678 | insertion      | C286-287 insertion                | 97 codon shift    | 24           |
| rv0678 | deletion       | G198 deletion                      | 67 codon shift    | 10           |
| rv0678 | insertion      | GAC346-348 insertion              | D116 insertion    | 5            |
| rv0678 | insertion      | G253 insertion                    | 85 codon shift    | 1            |
| rv0678 | insertion      | G430 insertion                    | 144 codon shift   | 1            |
| rv0678 | insertion      | A209 insertion                    | 70 codon shift    | 2            |
| rv0678 | deletion       | A205 deletion                      | 69 codon shift    | 1            |
| rv0678 | deletion       | AGACGGCGGGGGGATCAG187-203 deletion | 63 codon shift    | 1            |
| rv0678 | deletion       | A209 deletion                      | 70 codon shift    | 1            |
| rv0678 | deletion       | C471 deletion                      | 157 codon shift   | 1            |
| rv0678 | deletion       | G216 deletion                      | 73 codon shift    | 2            |
| rv0678 | deletion       | T451 deletion                      | 151 codon shift   | 2            |
| rv0678 | deletion       | G198 deletion                      | 67 codon shift    | 5            |
| rv0678 | deletion       | C349 deletion                      | 117 codon shift   | 1            |
| rv0678 | deletion       | C158 deletion                      | 53 codon shift    | 1            |
| rv0678 | deletion       | CG286-287 insertion               | 142 codon shift   | 1            |
| rv0678 | deletion       | T95 deletion                       | 32 codon shift    | 1            |
| rv0678 | deletion       | A415 deletion                      | 139 codon shift   | 1            |
| rv0678 | deletion       | G19 deletion                       | 7 codon shift     | 1            |
| rv0678 | deletion       | GQA51-53 deletion                 | 17 codon shift    | 1            |
| rv0678 | insertion      | G439 insertion                    | 147 codon shift   | 1            |
| rv0678 | insertion      | G286-287 insertion                | 97 codon shift    | 24           |
| rv0678 | insertion      | G198 insertion                    | 67 codon shift    | 10           |
| rv0678 | insertion      | GAC346-348 insertion              | D116 insertion    | 5            |
| rv0678 | insertion      | G253 insertion                    | 85 codon shift    | 1            |
| rv0678 | insertion      | G430 insertion                    | 144 codon shift   | 1            |
| rv0678 | insertion      | A209 insertion                    | 70 codon shift    | 1            |
| rv0678 | deletion       | A205 deletion                      | 69 codon shift    | 1            |
| rv0678 | deletion       | AGACGGCGGGGGGATCAG187-203 deletion | 63 codon shift    | 1            |
| rv0678 | deletion       | A209 deletion                      | 70 codon shift    | 1            |
| rv0678 | deletion       | C471 deletion                      | 157 codon shift   | 1            |
| rv0678 | deletion       | T451 deletion                      | 151 codon shift   | 2            |
| rv0678 | deletion       | G198 deletion                      | 67 codon shift    | 5            |
| rv0678 | deletion       | C349 deletion                      | 117 codon shift   | 1            |
| rv0678 | deletion       | C158 deletion                      | 53 codon shift    | 1            |
| rv0678 | deletion       | CG286-287 insertion               | 142 codon shift   | 1            |
| rv0678 | deletion       | T95 deletion                       | 32 codon shift    | 1            |
| rv0678 | deletion       | A415 deletion                      | 139 codon shift   | 1            |
| rv0678 | deletion       | G19 deletion                       | 7 codon shift     | 1            |
| rv0678 | deletion       | GQA51-53 deletion                 | 17 codon shift    | 1            |
| rv0678 | insertion      | G439 insertion                    | 147 codon shift   | 1            |
| rv0678 | insertion      | G286-287 insertion                | 97 codon shift    | 24           |
| rv0678 | insertion      | G198 insertion                    | 67 codon shift    | 10           |
| rv0678 | insertion      | GAC346-348 insertion              | D116 insertion    | 5            |
| rv0678 | insertion      | G253 insertion                    | 85 codon shift    | 1            |
| rv0678 | insertion      | G430 insertion                    | 144 codon shift   | 1            |
| rv0678 | insertion      | A209 insertion                    | 70 codon shift    | 1            |
| rv0678 | deletion       | A205 deletion                      | 69 codon shift    | 1            |
| rv0678 | deletion       | AGACGGCGGGGGGATCAG187-203 deletion | 63 codon shift    | 1            |
| rv0678 | deletion       | A209 deletion                      | 70 codon shift    | 1            |
| rv0678 | deletion       | C471 deletion                      | 157 codon shift   | 1            |
| rv0678 | deletion       | T451 deletion                      | 151 codon shift   | 2            |
| rv0678 | deletion       | G198 deletion                      | 67 codon shift    | 5            |
| rv0678 | deletion       | C349 deletion                      | 117 codon shift   | 1            |
| rv0678 | deletion       | C158 deletion                      | 53 codon shift    | 1            |
| rv0678 | deletion       | CG286-287 insertion               | 142 codon shift   | 1            |
| rv0678 | deletion       | T95 deletion                       | 32 codon shift    | 1            |
| rv0678 | deletion       | A415 deletion                      | 139 codon shift   | 1            |
| rv0678 | deletion       | G19 deletion                       | 7 codon shift     | 1            |
| rv0678 | deletion       | GQA51-53 deletion                 | 17 codon shift    | 1            |
| rv0678 | insertion      | G439 insertion                    | 147 codon shift   | 1            |
| rv0678 | insertion      | G286-287 insertion                | 97 codon shift    | 24           |
| rv0678 | insertion      | G198 insertion                    | 67 codon shift    | 10           |
| rv0678 | insertion      | GAC346-348 insertion              | D116 insertion    | 5            |
| rv0678 | insertion      | G253 insertion                    | 85 codon shift    | 1            |
| rv0678 | insertion      | G430 insertion                    | 144 codon shift   | 1            |
| rv0678 | insertion      | A209 insertion                    | 70 codon shift    | 1            |
| rv0678 | deletion       | A205 deletion                      | 69 codon shift    | 1            |
| rv0678 | deletion       | AGACGGCGGGGGGATCAG187-203 deletion | 63 codon shift    | 1            |
| rv0678 | deletion       | A209 deletion                      | 70 codon shift    | 1            |
TABLE 2 | Mutation analysis of 67 BDQ-resistant mutants of Mtb by PCR and Sanger analysis.

| Locus_tag_1 | Nucleotide change | Amino acid change | Locus_tag_2 | Nucleotide change | Amino acid change | Mutantcount |
|------------|------------------|------------------|------------|------------------|------------------|-------------|
| rv0678     | C214T            | R72W             | glpK       | G572 insertion   | 192 codon shift  | 1           |
| rv0678     | C214T            | R72W             | glpK       | WT               | WT               | 1           |
| rv0678     | T179C            | L60P             | glpK       | G572 insertion   | 192 codon shift  | 3           |
| rv0678     | T341C            | L114P            | glpK       | G572 insertion   | 192 codon shift  | 1           |
| rv0678     | T341C            | L114P            | glpK       | WT               | WT               | 1           |
| rv0678     | T350C            | L117P            | glpK       | G572 insertion   | 192 codon shift  | 1           |
| rv0678     | G194A            | G65E             | glpK       | G572 insertion   | 192 codon shift  | 1           |
| rv0678     | G304A            | A102T            | glpK       | G572 insertion   | 192 codon shift  | 1           |
| rv0678     | G15G             | D5E              | glpK       | G572 insertion   | 192 codon shift  | 1           |

rv0678 confer resistance to BDQ. Consistently, a spectrum of mutations in rv0678 in MDR-TB clinical isolates was identified, although most of them were not involved in BDQ resistance (Villellas et al., 2017). Additionally, the high frequency of mutations in rv0678 was not related to the prior use of BDQ (Villellas et al., 2017). Consistently, our results suggest that rv0678 mutations are frequent in the presence or absence of BDQ, and BDQ-resistant mutants were selected and enriched after BDQ utilization. A proportion of mutations in rv0678 identified in the present study were also found in clinical BDQ-resistant isolates (Villellas et al., 2017; Andres et al., 2020; Degiacomi et al., 2020; Nimmo et al., 2020a; Nimmo et al., 2020b), including G198, CC279–280, and TT279–280 deletions and G198, G199, A275, and G439 insertions and G120T substitution. In particular, 67 codon shifts caused by the G198 deletion and G198 and G199 insertions in rv0678 were the second most common mutations in our BDQ-resistant isolates, indicating that our study contributes to the identification of BDQ-resistant isolates in clinical settings. However, no mutations in atpE gene were found in the isolated mutants. We hypothesize that mutations in atpE result in relatively high-level resistance to BDQ (10–128-fold MIC); however, the BDQ concentration (0.5 µg/ml, eightfold MIC) used in our study is appropriate for the selection of low-level BDQ-resistant mutants. Consistently, mutations in rv0678 with a high frequency have been found in clinical isolates, whereas mutations in atpE have seldom been identified in clinical isolates (World Health Organization, 2019; Andres et al., 2020).

A previous study found that mutations in rv0678 cause cross-resistance to clofazimine both in vitro and in vivo (Hartkorn et al., 2014; Zhang et al., 2015; Xu et al., 2017). Accordingly, mutants harboring rv0678 G194A, G269C, G304A, T341C, and T365C mutations were also observed in clofazimine-resistant mutants (Zhang et al., 2015), suggesting that these mutants may be cross-resistant to clofazimine, and our results may provide new insights into clofazimine resistance. Whether the mutants identified in the present study were resistant to clofazimine warrants further investigation.
### Hitchhiking mutations identified in BDQ-resistant mutants of Mtb.

| Locus_tag_1 | Locus_tag_2 | Nucleotide change | Amino acid change | Locus_tag_3 | Nucleotide change | Amino acid change | Mutantcount |
|-------------|-------------|-------------------|-------------------|-------------|-------------------|-------------------|-------------|
| rv0678      | W42G (1);   | glnK C80G         | T27S              | cobQ2 and   | G223A and CTGTTG | G75S and 226 codon | 3           |
|             | 146 codon   |                   |                   | ptaA and gid| GTGATGTAGAT     | shift codon       |
|             | shift (1);  |                   |                   | and rv2426c | CCG655-671 insertion | and E121A and M1 deletion |
| rv0678      | 122 codon   |                   |                   |             | G223A and CTGTTG | G75S and 226 codon shift |
|             | shift (1);  |                   |                   |             | GTGATGATGTA      | shift codon       |
| rv0678      | 122 codon   |                   |                   |             | G655-671 insertion and A382C and CT | G75S and 226 codon shift |
|             | shift (1);  |                   |                   |             | AGAGGAAGACCGGATC | shift codon       |
| rv0678      | 147 codon   |                   |                   |             | G655-671 insertion and A382C | G75S and 226 codon shift |
|             | shift (1);  |                   |                   |             | G739A            | shift codon       |
| rv0678      | 62 codon    |                   |                   |             | G75S and 226 codon shift | E121A and K114N |
|             | shift (1) ; |                   |                   |             |                   |                   | 1           |
| rv0678      | 97 codon    |                   |                   |             |                   |                   | 1           |
|             | shift (10); |                   |                   |             |                   |                   | 2           |
|             | 67 codon    |                   |                   |             |                   |                   | 3           |
|             | shift (6);  |                   |                   |             |                   |                   | 2           |
|             | G103A (1);  |                   |                   |             |                   |                   | 3           |
|             | 53 codon    |                   |                   |             |                   |                   | 4           |
|             | shift (1);  |                   |                   |             |                   |                   | 5           |
|             | 151 codon   |                   |                   |             |                   |                   | 6           |
|             | shift (1);  |                   |                   |             |                   |                   | 7           |
| rv0678      | 97 codon    |                   |                   |             |                   |                   | 8           |
|             | shift (3) ; |                   |                   |             |                   |                   | 9           |
| rv0678      | 142 codon   |                   |                   |             |                   |                   | 10          |
|             | shift (1);  |                   |                   |             |                   |                   | 11          |
|             | 77 codon    |                   |                   |             |                   |                   | 12          |
|             | shift (1) ; |                   |                   |             |                   |                   | 13          |
| rv0678      | 97 codon    |                   |                   |             |                   |                   | 14          |
|             | shift (3) ; |                   |                   |             |                   |                   | 15          |
|             | 69 codon    |                   |                   |             |                   |                   | 16          |
|             | shift (1);  |                   |                   |             |                   |                   | 17          |
|             | 67 codon    |                   |                   |             |                   |                   | 18          |
|             | shift (2) ; |                   |                   |             |                   |                   | 19          |
|             | 77 codon    |                   |                   |             |                   |                   | 20          |
|             | shift (1) ; |                   |                   |             |                   |                   | 21          |
| rv0678      | 63 codon    |                   |                   |             |                   |                   | 22          |
|             | shift (1);  |                   |                   |             |                   |                   | 23          |
| rv0678      | 97 codon    |                   |                   |             |                   |                   | 24          |
|             | shift (2);  |                   |                   |             |                   |                   | 25          |
| rv0678      | L60P (1);   |                   |                   |             |                   |                   | 26          |
|             |             |                   |                   |             |                   |                   | 27          |
| rv0678      | 117 codon   |                   |                   |             |                   |                   | 28          |
|             | shift (1);  |                   |                   |             |                   |                   | 29          |
|             | 85 codon    |                   |                   |             |                   |                   | 30          |
| rv0678      | 97 codon    |                   |                   |             |                   |                   | 31          |
|             | shift (1);  |                   |                   |             |                   |                   | 32          |

(Continued)
| Locus_tag_1 | Locus_tag_2 | Nucleotide change | Amino acid change | Locus_tag_3 | Nucleotide change | Amino acid change | Mutantcount |
|------------|------------|-------------------|------------------|------------|------------------|------------------|-------------|
| rv0678 WT (3); | glpK | G572 insertion | 192 codon shift | lppB | C175A | H59N | 3 |
| rv0678 144 codon shift (1); | glpK | G572 insertion | 192 codon shift | n2823c | TCGCCGACA304-312 insertion | IAD102-104 insertion | 1 |
| rv0678 L142R (1); | glpK | G572 insertion | 192 codon shift | cobO2 and pitA and n2823c | G223A and CTGTGGGTGATCGTG | G75S and 226 codon shift and IAD102-104 insertion | 1 |
| rv0678 L142R (1); | glpK | WT | WT | cobO | G223A | G75S | 1 |
| rv0678 R94W (1); | glpK | WT | WT | cobO and n2426c | G223A and CTAGAGGAGACCGAGATCGTG | G75S and M1 deletion | 1 |
| rv0678 142 codon shift (1); | glpK | G572 insertion | 192 codon shift | cobO and n128B | G223A and CTGTGGGTGATCGTG | G75S and 226 codon shift and H59N | 1 |
| rv0678 32 codon shift (1); | glpK | G572 insertion | 192 codon shift | n0405 | G2276-2276 deletion | G75S and 226 codon shift | 1 |
| rv0678 67 codon shift (1); | glpK | G572 insertion | 192 codon shift | cobO2 and lppB | G223A and C175A | G75S and H59N | 1 |
| rv0678 70 codon shift (1); | glpK | G572 insertion | 192 codon shift | n2722 and lppB | G223A and CTGTGGGTGATCGTG | G75S and 226 codon shift and Y-G | 1 |
| rv0678 67 codon shift (1); | glpK | G572 insertion | 192 codon shift | trcR and lppB | G223A and C175A and C629 insertion | G75S and H59N and 211 codon shift | 1 |
| rv0678 73 codon shift (1); | glpK | G572 insertion | 192 codon shift | cobO and lppB and n3785 | G223A and CTGTGGGTGATCGTG | G75S and 226 codon shift and P233L | 1 |
| rv0678 L142R (1); | glpK | G572 insertion | 192 codon shift | cobO2 and pitA and n1723 | G223A and CTGTGGGTGATCGTG | G75S and 226 codon shift and P233L | 1 |
| rv0678 L40F (1); D116 insertion (1); 67 codon shift (1); | glpK | WT | WT | WT | WT | WT | 3 |
Although mutations in *atpE, rv0678*, and *pepQ* confer major resistance to BDQ, the mechanism of resistance to BDQ for a portion of isolates identified *in vitro* and in clinical settings remains unknown, as they had WT *atpE, rv0678*, and *pepQ* (Andres et al., 2020). It is likely that there are other unclarified mechanisms of resistance to BDQ. Here, we identified a new gene, *glpK*, and an insertion mutation in *glpK* (G572 insertion) that resulted in a frameshift and loss of function, leading to BDQ resistance. GlpK, encoded by *rv3696c*, is a critical enzyme for the uptake and metabolism of glycerol and phosphates. Glycerol is a potential source of carbon and energy for *Mtb* (Nourozi et al., 2015). A recent report showed that a frameshift mutation in the 7C homopolymeric tract (HT) sequence (CCCCCCCCCC66-572) of *glpK*, which is equal to the G572 insertion identified in our study, conferred drug tolerance to isoniazid, rifampicin, moxifloxacin, and ethambutol (Safi et al., 2019; Sun et al., 2019). These frameshift mutations in *glpK* lower *Mtb* growth under certain culture conditions (glycerol-containing culture conditions) but enhance survival after exposure to different anti-TB agents (Safi et al., 2019; Sun et al., 2019). Moreover, *glpK* mutants can revert back to WT *glpK* by introducing additional insertions and deletions in the same *glpK* HT region, thus reversing the slow growth (Safi et al., 2019). The reversible process between frameshift mutations and the WT in the 7C HT sequence of *glpK* conferring reversible drug tolerance during anti-TB therapy facilitates the survival of bacteria *in vivo*, suggesting that similar circumstances may also exist in BDQ resistance. Here, our results showed that mutants harboring the G572 insertion in *glpK* were associated with BDQ resistance (Table 4). However, mutants with both G572 insertion in *glpK* and *rv0678* did not increase the MIC to BDQ, suggesting that they have no synergistic or superimposed effects on BDQ resistance (Table 4). According to Safi’s study, we hypothesized that the frameshift mutation in *glpK* conferring resistance to BDQ caused reduced *Mtb* growth (Safi et al., 2019). We found that a large proportion of the isolated mutants formed small colonies with slow growth on 7H10 agar plates (data not shown).

Interestingly, irreversible frameshift mutations in HTs is caused by slipped-strand mispairing errors during proliferation, and there is a high-proportioned HT variants in bacteria with DNA mismatch repair defect, including *Mtb* (Streisinger and Owen, 1985; Cole et al., 1998; Mizrahi and Andersen, 1998; Canceill et al., 1999; Parkhill et al., 2000; Bayliss et al., 2008; Bayliss, 2009), indicating that the poly-G:C and poly-A:T tracts within the *Mtb* genome might have invertible insertion and deletion mutations during proliferation, such as the G572 insertion in the 7C HT sequence identified in the present study. Moreover, mutants with frameshift mutations in *glpK* in an unstable state have been detected in the sputum of patients with TB (Black et al., 2015; Trauner et al., 2017). Worse still, free glycerol is present in human plasma and *Mtb*-infected mouse lung tissue, and Safi et al. have demonstrated that the *in vivo* environment offers positive selection for *glpK* mutations (Safi et al., 2019). Therefore, it should bring to the forefront that frameshift mutation in *glpK* caused by mismatch repair deficiency confers BDQ resistance in clinical settings. Together, we revealed a previously undetermined role of *glpK* in low-level BDQ resistance.

In summary, we characterized 168 mutants resistant to BDQ and found that mutations in *rv0678* confer the primary mechanism of BDQ resistance. Importantly, we identified a new gene (*glpK*) involved in BDQ resistance. Further studies are needed to address the role of *glpK* by constructing *glpK* knockout and complement strains. Our study offers new insights and valuable information that will contribute to rapid identification of BDQ-resistant isolates in clinical settings.

### DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

### AUTHOR CONTRIBUTIONS

QG and JB performed the experiments. QG, JB, QL, TY, ZhoW, ZhaW, LL, and GZ analyzed the data. QG, JB, and GZ designed the study and wrote the paper. QL, TY, ZhoW, ZhaW, and LL reviewed the paper and supervised the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.807095/full#supplementary-material
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