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MECHANISMS OF RESISTANCE

Emergence of Canonical and Noncanonical Genomic Variants following *In Vitro* Exposure of Clinical *Mycobacterium tuberculosis* Strains to Bedaquiline or Clofazimine

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ABSTRACT In *Mycobacterium tuberculosis*, bedaquiline and clofazimine resistance occurs primarily through *Rv0678* variants, a gene encoding a repressor protein that regulates *mmpS5/mmpL5* efflux pump gene expression. Despite the shared effect of both drugs on efflux, little else is known about other pathways affected. We hypothesized that *in vitro* generation of bedaquiline- or clofazimine-resistant mutants could provide insight into additional mechanisms of action. We performed whole-genome sequencing and determined phenotypic MICs for both drugs on progenitor and mutant progenies. Mutants were induced through serial passage on increasing concentrations of bedaquiline or clofazimine. *Rv0678* variants were identified in both clofazimine- and bedaquiline-resistant mutants, with concurrent *atpE* SNPs occurring in the latter. Of concern was the acquisition of variants in the F420 biosynthesis pathway in clofazimine-resistant mutants obtained from either a fully susceptible (*fbiD*: del555GCT) or rifampicin mono-resistant (*fbiA*: 283delTG and T862C) progenitor. The acquisition of these variants possibly implicates a shared pathway between clofazimine and nitroimidazoles. Pathways associated with drug tolerance and persistence, F420 biosynthesis, glycerol uptake and metabolism, efflux, and NADH homeostasis appear to be affected following exposure to these drugs. Shared genes affected by both drugs include *Rv0678*, *glpK*, *nuoG*, and *uvrD1*. Genes with variants in the bedaquiline resistant mutants included *atpE*, *fadE28*, *truA*, *mmpL5*, *glnH*, and *pks8*, while clofazimine-resistant mutants displayed *ppsD*, *fbiA*, *fbiD*, *mutT3*, *fadE18*, *Rv0988*, and *Rv2082* variants. These results show the importance of epistatic mechanisms as a means of responding to drug pressure and highlight the complexity of resistance acquisition in *M. tuberculosis*.

KEYWORDS clofazimine, bedaquiline, *Mycobacterium tuberculosis*, *in vitro* mutants, genetic signatures, resistance, canonical, noncanonical variants

Bedaquiline and clofazimine are novel and repurposed antituberculosis (anti-TB) drugs, respectively, that offer promising options to treat and alleviate TB disease. In particular, they are used as therapeutics for drug-resistant TB, which is more challenging to diagnose and treat compared to susceptible disease forms. Bedaquiline and clofazimine usage has increased since WHO approved both drugs for the treatment of rifampicin-resistant tuberculosis (TB) (1). At a juxtaposition to this increased usage is the lack of a rapid genotypic drug susceptibility test (DST) for these two drugs (2). Although variants in the *Rv0678* gene, encoding a repressor protein, which affects the

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expression of the *mmpS5/mmpL5* efflux pump (3 to 5), are associated with both clofazimine and bedaquiline resistance, there is a lack of understanding of all genetic components involved in resistance for these two drugs. This is due to the rarity of resistant *Mycobacterium tuberculosis* isolates available for investigation and the lack of agreement and availability of phenotypic DST associated with genotypic DST data (6); the presence of both wild-type and variant forms of *Rv0678* (heteroresistance) observed in clinical isolates (7); and the inconsistency of the association of insertions and deletions (indels) in *Rv0678* and bedaquiline resistance (8).

While bedaquiline is known to target subunit C of the ATP synthase, encoded by *atpE*, there are a limited number of clinical strains with *atpE* variants (9). Other nontarget genes for bedaquiline include *Rv1979c* (a putative permease) and *pepQ* (cytoplasmic peptidase) (10). Clofazimine resistance has been loosely associated with the latter two genes (3, 10). Furthermore, clofazimine has been shown to be reduced enzymatically by the NADH:quinone oxidoreductase (encoded by *ndh2*), but to date no clofazimine-resistant *ndh* mutants (or mutants with genetic variants in redox pathways) have been reported (11, 12). In the 2021 WHO catalogue of drug-resistance-associated mutations, *Rv0678*, *pepQ*, *mmpS5*, and *mmpL5* genes are considered to be tier 1, meaning that these genes are considered to most probably contain resistance-conferring variants for both bedaquiline and/or clofazimine (6). *Rv1979c* is a tier 2 gene, which has a reasonable probability of containing resistance-conferring variants (6).

In this study, we aimed to identify whether *in vitro* exposure of a set of progenitor clinical *M. tuberculosis* isolates to either bedaquiline or clofazimine leads to the accumulation of variants in addition to *Rv0678* variants. We investigated the phenotypic and genotypic characteristics of mutants compared to the baseline characteristics of the progenitor strains.

RESULTS

Each of the selected clinical isolates was genetically characterized by whole-genome sequencing (WGS) to confirm their lineage classification as well as their genetic drug susceptibility (gDST) pattern (see Tables S1 and S2 in the supplemental material). WGS confirmed the presence of a single strain in each of the clinical isolates. WGS analysis metrics showed a median depth of coverage of 35× and no evidence of contamination (based on the high percentage of mapped reads; Table S2). WGS of the progenitor strains revealed that the M2 strain exhibited additional resistance to pyrazinamide, streptomycin, and ethambutol; the M3 strain had additional pyrazinamide resistance, and the M1 strain had additional streptomycin resistance (Table S1).

Induction of bedaquiline resistance. Bedaquiline-resistant mutants were created by serial passage of H37Rv, and each clinical strain on increasing concentrations of each drug (Table S3). Following five passages on bedaquiline-containing media, a range of the highest growth-permitting concentrations was observed for the respective clinician strains and H37Rv (0.25 to 4 μg/mL; Table S3). Importantly, all bedaquiline-resistant mutants displayed MICs above the critical concentration (CC) of 1 μg/mL (Table 1). WGS identified variants in *atpE* and/or *Rv0678*, which included a combination of nonsynonymous substitutions and/or indels (Table 1). This suggests that the serial selection process selected populations with a single variant (S1, R1, M2), multiple variants conferring bedaquiline resistance (S2, M3, H37Rv), or multiple clones with different variants conferring bedaquiline resistance (M1) (Table 1).

The bedaquiline-resistant culture derived from the S1 strain (*Rv0678* frameshift) showed significantly higher growth rates compared to its progenitor strain ($p < 0.01$) (Table 1, Fig. S1). Interestingly, the bedaquiline-resistant culture derived from the H37Rv, S2, and M3 strains (with *atpE* variants) all displayed lower growth rates compared to their progenitor strains. From these, only the bedaquiline-resistant culture from the H37Rv strain displayed a significant difference in the growth rate compared to its progenitor strain ($P < 0.01$). Bedaquiline-resistant culture with only *Rv0678*

TABLE 1 Comparison of baseline phenotypic data for progenitor strains compared to phenotypic (MIC and length of lag phase and growth rates) and genotypic data for bedaquiline-resistant mutants[a]

	Bedaquiline MIC in μg/mL (MGIT)		Variants acquired						Avg growth rate (hrs)	
			Rv0678		*atpE*		Other			
Strain	Base line	Mutant	NT (AA)	Freq.	NT (AA)	Freq.	Gene: NT (AA)	Freq.	Base line	Mutant
H37Rv	≤0.125	>8	T2C (Val1Ala)	0.46	A83G (Asp28Gly)	0.65	*fadE28:* C578T (Ala193Val)	1.00	19	41 (*)
					G183C (Gly61Asp)	1.00	*ponA2:* G1614C (syn)	0.18		
S1	0.5	8	90_91insA	1.00			*truA:* 8insG	0.76	34	24 (*)
S2	0.5	>8	G74A (Gly25Asp)	1.00	C188T (Ala63Val)	1.00			23	31
R1	≤0.125	8	T416G (Met139Arg)	0.97			*mmpL5:* A2773G (Met925Val)	0.96	35	36
							glnH: G712C (Ala238Pro)	1.00		
							pks8: C505G (Leu169Val)	0.93		
							glpK: 573insC	1.00		
							Rv2326c: C147T (syn)	1.00		
M1	1	4	G203A (Ser68Asn)	0.25			*nuoG:* G1246C (Ala416Pro)[b]	0.59	31	28
			T374C (Leu125Pro)	0.23			*glpK* 573insC	0.96		
			431delAT	0.18						
M2	≤0.125	4	T461C (Leu154Pro)	1.00			*uvrD1:* T1991C (Met664Thr)	1.00	28	23
M3	1	8	66_67insT	1.00	G183T (Glu61Asp)	1.00	114 bp insertion between *Rv3680* and *whiB4*	1.00	46	76

[a]NT indicates the nucleotide change, and AA indicates the amino acid change. Freq. indicates the allele frequency for the variant. Statistically significant differences between mutant and baseline for growth rates (hours, hrs) are indicated by *, $P < 0.05$. Synonymous variants indicated in parentheses.

[b]*nuoG* variant present in progenitor at lower frequency.

TABLE 2 Comparison of baseline phenotypic data for progenitor strains compared to phenotypic (MIC and average growth rates) and genotypic data for clofazimine-resistant mutants[a]

	CFZ MIC in μg/mL (MGIT)		Variant acquired				Avg growth rate (hrs)	
			Rv0678		Other			
Strain	Baseline	Mutant	NT (AA)	Freq.	Gene: NT (AA)	Freq.	Baseline	Mutant
H37Rv	0.125	4	211insC	1.00	*ppsD:* T3518C (Leu1173Pro);	1.00	19	35 (**)
					fbiD: del555GCT	0.51		
					Rv3049c: C549T (syn)	1.00		
S1	0.5	4	192_193delG	0.61	*atsB*: C591T (syn)	0.77	34	42
			T167C (Leu56Pro)	0.17	*mutT3: T155A (L52Q)*	0.2		
					fadE18: G608T (R203D)	0.22		
					A607C (R203D)	0.22		
S2	0.5	2	192_193delG	0.49			23	36
			A208G (Asn70Asp)	0.23				
			G404C (Arg135Pro)	0.24				
R1	0.5	4	A65C (Gln22Pro)	0.48	*Rv0988:* C493G (Arg165Gly)	0.57	35	84
					fbiA: 283delTG	0.39		
			166_177del ACTGGCGACGGCG	0.40	*fbiA:* T862C (Trp288Arg)	0.31		
					glpK: 573insC	1.00		
M1	0.5	4	T461C (Leu154Pro)	0.31	*nuoG:* G1246C (Ala416Pro)[b]	0.95	31	33
			274insA	0.2	*glpK:* 573insC	1.00		
M2	0.5	2	C251T (Ala84Val)	0.51	*uvrD1:* T1991C (Met664Thr)	0.79	28	23 (*)
			G404C (Arg135Pro)	0.09	*Rv2082:* G2083A (Ala695Thr)	0.18		
			465insC	0.21				
M3	0.5	4	T128C (Leu43Pro)	0.17			46	49
			T131C (Leu44Pro)	0.16				
			G215A (Arg72Gln)	0.2				

[a]Synonymous variants indicated in brackets (syn). NT indicates the nucleotide change, and AA indicates the amino acid change. Freq. indicates the allele frequency for the variant. Statistically significant differences between mutant and baseline for growth rates (hours, hrs) are indicated by * and **, which represent *P*-values of <0.05 and 0.01, respectively.

[b]*nuoG* variant present in progenitor at lower frequency.

nonsynonymous variants (M1, M2, and R1) all displayed similar growth rates compared to their progenitor strains (Table 1, Fig. S1).

Repeated exposure to bedaquiline also selected for variants outside *atpE* and *Rv0678*. Briefly, variants were identified in *fadE28* (acyl coA dehydrogenase), *truA* (uracil hydrolase), *glnH* (glutamine-binding lipoprotein), *uvrD1* (ATP-dependent DNA helicase), *Rv2366c* (transmembrane protein), *nuoG* (NADH dehydrogenase), *glpK* (glycerol kinase), and *mmpL5* (transmembrane transport protein) (Table 1). The frequency at which these variants appeared ranged from 59% to 100%. Interestingly, two of the mutants derived from the R1 and M1 strains acquired an identical indel in the *glpK* gene. The *mmpL5* variant (A2773G) was also identified in the bedaquiline-resistant mutant derived from the R1 strain. We also identified a synonymous mutation in the bedaquiline-resistant mutant from the R1 strain in the *Rv2326c* gene (C147T) (Table 1).

Induction of clofazimine resistance. Following four passages on clofazimine-containing media, the highest growth-permitting concentrations observed for the seven strains was either 1 μg/mL (S2, M1, M3) or 2 μg/mL (H37Rv, S1, R1, M2) (Table S4). WGS showed that variants in *Rv0678* were responsible for clofazimine resistance. With the exception of H37Rv, variant frequencies were less than 61%, indicative of heteroresistance (Table 2). All of the clofazimine-selected cultures displayed MICs higher than the baseline MICs for clofazimine and above the CC of 1 μg/mL, an indication of resistance (Table 2).

In addition to variants in *Rv0678*, WGS identified nonsynonymous single-nucleotide variants in *ppsD* (polyketide synthase), *fbiD* (*Rv2983*, conserved hypothetical alanine-rich protein), *Rv0988* (conserved exported protein), *fbiA* (F420 biosynthesis protein), *glpK* (glycerol kinase), *uvrD1* (ATP-dependent DNA helicase), *Rv2082* (conserved hypothetical protein), *nuoG* (NADH dehydrogenase), and *glpK* (glycerol kinase) (Table 2). This analysis also identified synonymous mutations in the clofazimine-selected cultures

H37Rv and S1 strains in the *Rv3049c* (C549T) and *Rv3299c* genes (C591T), respectively (Table 2).

The H37Rv clofazimine-resistant culture displayed a significantly lower growth rate compared to the progenitor strain ($P < 0.01$), while the clofazimine-resistant cultures M1 and R1 displayed significantly higher growth rates ($P < 0.01$) (Table S5 and Fig. S1).

Intragroup comparison for bedaquiline and clofazimine mutants. Both bedaquiline- and clofazimine-resistant mutant cultures derived from the M1 strain acquired identical *Rv0678* variants, i.e., T461C (Leu154Pro), demonstrating a cross-resistance through this variant. Interestingly, both bedaquiline- and clofazimine-resistant mutant cultures derived from the M1 and M2 strains acquired identical *uvrD1* (T1991C: Met664Thr) and *glpK* variants (573insC), respectively (Tables 1 and 2).

DISCUSSION

Bedaquiline and the repurposed drug clofazimine are now considered core agents for treatment of drug-resistant TB. The introduction of standardized DST for analysis of phenotypic resistance to both bedaquiline and clofazimine represents a progressive step toward employing regimens that are effective (2). While rapid genotypic tests and the use of WGS can be used to detect *Rv0678* variants associated with bedaquiline and clofazimine resistance, the release of the WHO 2021 catalogue of drug-resistance-associated mutations 3 years after endorsing bedaquiline for treatment of drug-resistant TB revealed the paucity of complementary phenotypic and genotypic data for both bedaquiline and clofazimine resistance (6). This study adds to the current body of knowledge, and the results enrich the information contained in existing catalogues (6, 13 to 15) and shed light on the complexity of pathways involved in resistance acquisition in *M. tuberculosis*.

We identified a single base insertion at position 573 of the *glpK* gene, which extended the length of the homopolymeric tract (position 566 to 572) from 7C to 8C. The *glpK* gene encodes glycerol 3-phosphotransferase, which is essential for glycerol uptake and metabolism. This variant was found in mutant cultures derived from both Beijing and Latin American Mediterranean (LAM) backgrounds, confirming the finding from Safi et al. that transient tolerance can occur in a wide range of phylogenetic lineages and display the same effect (16). Furthermore, regardless of the drug used for induction (i.e., bedaquiline or clofazimine), the same strains were affected (R1 and M1). Interestingly, despite all the strains being placed under the same experimental conditions, only these two acquired *glpK* frameshift variants. It is unclear if this is due to the genetic background of these specific strains or the labile nature of *glpK* variants. While homopolymers are problematic and error prone in sequencing, the frequency for these variants was >95% for both mutants, and the presence of these variants has also been confirmed by other studies (17).

A similar phenomenon was observed with both clofazimine- and bedaquiline-resistant cultures obtained from the M2 strain, i.e., the acquisition of identical *uvrD1* variants (T1991C [Met664Thr]). *UvrD1* encodes a DNA helicase that unwinds G-quadruplex DNA secondary structures (in an ATP-dependent manner) to maintain genome integrity. While this gene is not essential for mycobacteria survival (unlike *uvrD2*), it has also been shown to be involved in pathogenesis and persistence (18). *UvrD1* deletion mutants have been found to be hypersusceptible to certain reactive oxygen intermediates and reactive nitrogen intermediates (18). Although the reduction of clofazimine leads to the formation of reactive oxygen species (ROS) (11), it is unclear if the variant identified in *uvrD1* would ameliorate the effect of ROS in the bacteria. Additionally, ROS production was not found to be increased by bedaquiline in other studies (19).

The progenitor M1 strain harbored a low-frequency (<30%) *nuoG* variant. Exposure to either bedaquiline or clofazimine resulted in a mutant population with a high-frequency *nuoG* variant, particularly for the clofazimine mutant population (0.95 versus 0.59). *nuoG* is a virulence gene belonging to the *nuo* operon, containing 14 genes, which codes for NADH dehydrogenase type I (Ndh-1) (20). The original hypothesis was that clofazimine requires activation *via* Ndh-2 (11), with limited data pointing to Ndh-1

in *M. tuberculosis* (12, 21, 22). Ndh-2 is the primary dehydrogenase used by *M. tuberculosis* and is essential for the survival of the bacteria, while Ndh-1 is a proton-pumping dehydrogenase, found to be nonessential for bacterial growth (12). Both of these enzymes appear to play active roles in maintaining NADH homeostasis (12). Interestingly, *M. leprae* only has the *nuoN* pseudogene, and the entire *nuo*-operon is deleted in this mycobacterial species (23), which may explain the efficacy of clofazimine as a leprosy drug having only one NADH dehydrogenase to target. Alternatively, the role of *nuoG* in these bedaquiline and clofazimine mutants may be explained by interference with the proton motive force (through bedaquiline affecting ATP synthase or clofazimine affecting Ndh-2), which may impact the expression of certain genes and the preferred use of different enzymes (proton-pumping Ndh-1 versus nonproton pumping Ndh-2) accordingly (21).

Rv0678 variants were the most common variants in both bedaquiline- and clofazimine-resistant mutants derived from all seven strains. These variants were never observed on their own, but rather in combination with other *Rv0678* variants, other *atpE* variants, or other variants in noncanonical pathways. When multiple low-frequency *Rv0678* variants occurred, we analyzed the alignments, and these appeared to belong to distinct subpopulations within the sequenced mutant population. In the case of high-frequency *Rv0678* and *atpE* variants, we previously showed that *atpE*-related resistance is likely the final step in high-level bedaquiline resistance (24), which could explain the lack of occurrence of independent *atpE* variants. However, an additional factor to consider is the genetic background of the strain, which appears to influence the variant acquired, as our previous study showed that fully susceptible or mono-resistant *M. tuberculosis* reference strains used for bedaquiline mutant generation display independent *atpE* variants (25). The acquisition of an *atpE* variant could also be associated with a concurrent loss of fitness. The lower growth rate in the bedaquiline-resistant mutant cultures that acquired *atpE variants* (i.e., from the H37Rv, S2, and M3 strains) could be evidence for this; however, a statistically significant difference was not shown for the latter two strains. The acquisition of variants other than *atpE* and *Rv0678* could be an indication of the complex number of pathways associated with resistance to these two drugs.

Cell-wall biosynthesis pathways have been previously postulated to accommodate shifts due to resistance (26). In this study, we show that multiple genes are involved, in particular those used to maintain homeostasis along the cell membrane. However, other than the genes mentioned above, all variants detected in other genes were single events and did not overlap, neither between strains nor between drugs. Of concern, however, were the *fbiD* and *fbiA* variants identified in two distinct clofazimine-resistant mutants derived from the H37Rv and R1 strains. FbiD (*Rv2983*) is a phosphoenolpyruvate (PEP) guanylyltransferase that synthesizes the phosphoenolpyruvyl moiety, which is subsequently transferred to F0 by FbiA (27). *fbiA* encodes a 2-phospho-L-lactate transferase, which transfers the lactyl phosphate moiety of lactyl-2-diphospho-5-guanosine to 7,8-didemethyl-8-hydroxy-5-deazariboflavin in the F420 biosynthesis pathway (28, 29). While *fbiA* is not essential for *M. tuberculosis in vitro* growth, it was found that variants in the *fbiA* gene alter the production of F420 (30). Both *fbiA* variants identified in this study (T862C and 283delTG) appear in close proximity to those previously reported in delamanid- and pretomanid-resistant isolates (30). The enzymes involved in the F420 biosynthesis pathway are well known to be involved in resistance to nitroimidazoles (delamanid and pretomanid) (31). Additionally, *fbiD* mutants have been found to be cross-resistant to both pretomanid and delamanid (32). While it has been previously demonstrated that an F420-deficient pretomanid-resistant *fbiD* mutant is hypersusceptible to clofazimine (32), to our knowledge, this is the first study that demonstrates the acquisition of a variant in fbiD following exposure to clofazimine. Another study, by Waller et al. (33), has shown the selection of both *fbiA* and *fbiC* variants following clofazimine exposure and confirmed the role of genetic variants in the F420 biosynthesis pathway (besides *ddn*) with low-level clofazimine cross-resistance. Currently, with pretomanid and bedaquiline being used together in the BPaL regimen, investigating this association is critical to ensure protection of the TB drug arsenal.

Finally, the *mmpL5* gene encodes the protein involved in the mmpS5-mmpL5 efflux pump, which is regulated by *mmpR* (encoded by *Rv0678*) (3). The *mmpL5* variant acquired by the R1 strain in the bedaquiline-resistant mutant was not observed in the progenitor strain and is therefore unlikely to be phylogenetically relevant. However, this variant cooccurs with an *Rv0678* variant (T416G), and the WHO mutant catalog has classified an *Rv0678* variant at this nucleotide position (T416C) as having uncertain significance as it was only previously identified in two bedaquiline-sensitive strains (6). This could mean that the *mmpL5* variant could be responsible for the observed MIC increase or resistance or could play a role in compensation (if the *Rv0678* variant is key to the acquired resistance observed).

The use of a small sample size is a key limitation in this study as the vast diversity of the noncanonical variants identified also now require further confirmation. The presence of overlapping variants between bedaquiline- and clofazimine-resistant mutant cultures could be a result of the strain becoming culture adapted or an undetected underlying population (particularly in the case of the underlying *nuoG* population) being specifically selected due to serial drug exposure. Furthermore, the use of mutant populations rather than single clones (as evidenced by variant frequencies from WGS data) could impact the findings from the growth-rate studies. However, purifying to a single clone level would not show the diversity of mutations that can arise following induction on drug-containing media. Future research should focus on phenotypic drug susceptibility testing for delamanid and pretomanid for the *fbiA* and *fbiD* mutants. Additionally, WGS after the final passage could be compared with WGS analysis following each passage to compare the accumulation of resistance. Finally, comprehensive genomic and possibly transcriptomic analysis within these resistant *M. tuberculosis* strains could be performed to further elucidate mechanisms of action for these drugs.

In conclusion, we show the potential of *in vitro* resistance induction to both bedaquiline and clofazimine without apparent loss of fitness (especially in the presence of *Rv0678* variants), which is highly concerning. While the limited number of isolates in this study may provide only a cursory glance, it is plausible that the genetic background may influence the type of variant selected for and the degree of impact on associated pathways, as seen in larger studies focused on *Rv0678*-related resistance (34). There appears to be a putative link between transient tolerance (*glpK*), *nuoG* and *uvrD1* genes, and bedaquiline and clofazimine resistance. The unique genes implicated in the resistance acquisition for bedaquiline and clofazimine suggest that *M. tuberculosis* uses a complex involvement of pathways required to maintain fitness to accommodate the shift from a susceptible to resistant genotype.

MATERIALS AND METHODS

All experimental work was done in the BSL3 laboratory of the National TB Reference Laboratory and WHO TB Supranational Reference Laboratory (South Africa). The use of deidentified clinical *M. tuberculosis* strains was approved by the Research Ethics Committee (University of Pretoria, Faculty of Health Sciences—Ref: 309/2016). The clinical isolates were collected during routine surveillance with drug susceptibility and, in some cases, spoligotyping data. Fig. 1 describes the experimental workflow for clinical sample set selection, *in vitro* mutant generation, and global genomic analysis.

Sample set selection. Six clinical strains that belonged to either T-type, LAM, X-type, or Beijing lineages and that had different drug-susceptibility profiles (fully susceptible, rifampicin-mono resistant, or multidrug resistant) were selected. An ATCC 27294 *M. tuberculosis* H37Rv reference strain was also included as a control strain. Each strain was cultured using the Bactec MGIT960 automated liquid culture system (Becton, Dickinson Diagnostic Systems [BD Biosciences], Sparks, MD, USA). This was followed by purity determinations (blood agar and ZN staining), baseline whole-genome sequencing (WGS), and baseline susceptibility testing for bedaquiline and clofazimine in MGIT (mycobacterial growth indicator tube) media (25, 35). Strains used are abbreviated according to their susceptibility profiles: S (susceptible), R (rifampicin-mono resistant), and M (multidrug resistant). The strains were S1 (referring to the Beijing susceptible strain), S2 (referring to the T-type susceptible strain), R1 (referring to the Beijing rifampicin-mono resistant strain), M1 (referring to the LAM MDR strain), M2 (referring to the T-type MDR strain), and M3 (referring to the X-type MDR strain).

Mutant generation. Bedaquiline- and clofazimine-resistant *M. tuberculosis* mutants were generated as previously described (25). Briefly, bacterial cell suspensions of actively growing isolates with a turbidity equivalent to a McFarland 1.0 standard were inoculated on five Middlebrook 7H10 agar plates (supplemented with OADC) with different bedaquiline (Janssen Therapeutics, Titusville, NJ, USA) concentrations (range of

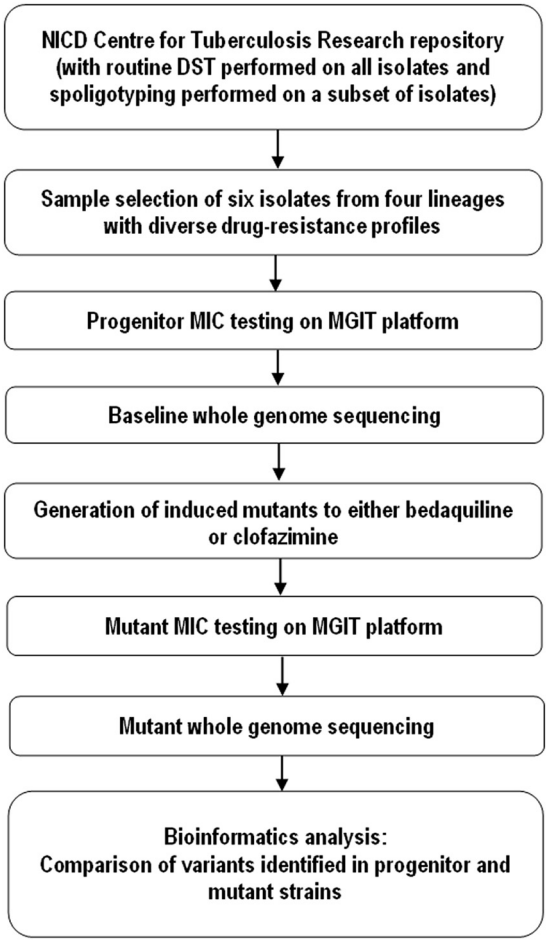

FIG 1 Experimental workflow for progenitor sample set characterization, mutant generation, and phenotypic and genotypic mutant characterization. NICD, National Institute for Communicable Diseases; DST, drug susceptibility testing; MGIT, mycobacteria growth indicator tube.

0.004 to 0.06 μg/mL) and a drug-free control plate. For the second passage, growth from the plate with the highest bedaquiline concentration was used to inoculate (McFarland 1.0 cell suspension) four plates (a drug-free control plate, a plate containing the growth-permitting concentration, and plates containing either 2- or 4-fold higher bedaquiline concentrations). Passaging was continued for a total of five passages, after which confluent growth was scraped off from the plates and used for subsequent MIC determinations. No further passaging was performed on plates containing $>$4 μg/mL bedaquiline. Clofazimine-resistant mutants were created using the same methodology, using a starting range of 0.125 to 0.5 μg/mL clofazimine (C8895, Sigma-Aldrich Co., St. Louis, USA). Passaging was continued for a total of four passages. For each passage, plates were incubated at 37°C until sufficient growth appeared for the creation of a McFarland 1.0 cell suspension (minimum 21 and maximum 28 days).

Baseline and mutant phenotypic characterization. Growth from MGIT tubes subcultured from each clinical isolate was used for MIC determinations and DNA extraction (WGS) for baseline characterization. Ten microliters of a suspension created from confluent growth from the plates containing the highest drug concentration from either the final passage or the last control passage were respectively inoculated into MGIT tubes for a drug-free passage to prepare the inoculum for subsequent MIC determinations using the Bactec MGIT960 platform and DNA extraction.

Determination of MIC values. Bedaquiline (Janssen Therapeutics, Titusville, NJ, USA) and clofazimine (Ref: C8895, Sigma-Aldrich Co., St. Louis, USA) were formulated in DMSO (Ref: 41639, Sigma-Aldrich Co.) to stock concentrations of 1 mg/mL and maintained at −20°C (max: 3 months). Twofold dilutions with a final concentration ranging from 8 to 0.125 μg/mL (bedaquiline) and 4 to 0.06 μg/mL (clofazimine) were prepared from the stock solutions. An 8 μg/mL clofazimine concentration could not be included as the color of the drug solution interfered with the florescent detection of the Bactec MGIT960 instrument. MIC determinations were performed as previously described (25, 35). A 1:5 dilution of a 3- to 5-day positive liquid culture was used to inoculate (500 μL) seven MGIT tubes containing the above-described range of bedaquiline or clofazimine concentrations. A further 1:100 dilution of the 1:5 suspension was used to inoculate (500 μL) a drug-free MGIT control tube. A H37Rv strain was included in each batch of bedaquiline and clofazimine MIC determinations conducted. Tubes were incubated

until the growth control reached 400 growth units (GUs) or for a maximum of 28 days. The MIC value was defined as the lowest drug concentration at which bacterial growth was inhibited (36).

Baseline and mutant genotypic characterization. Genomic DNA extraction was performed using the on-board generic protocol on the NucliSENS easyMag (bioMérieux, Marcy-l'Étoile, France). DNA concentrations were determined using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) with the Qubit dsDNA High Sensitivity (HS) assay kit (Life Technologies). Paired-end libraries were prepared using the Nextera XT DNA library kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol with a modified library normalization step (26). WGS was carried out using an Illumina MiSeq 2 × 300 bp V3 cartridge on the Illumina MiSeq platform.

Raw sequence data were analyzed as previously described (37). Briefly, reads were trimmed with Trimmomatic (38) and aligned to the *M. tuberculosis* H37Rv reference genome (GenBank NC000962.3) with BWA (39), SMALT (40), and Novoalign (Novocraft). Genomic variants (single nucleotide variants and insertions and deletions) identified in all three alignments with SAMTools (41) and the Genome Analysis Toolkit (42) were considered high-confidence variants. Pairwise comparison of the variants identified in progenitor isolates and their corresponding mutants were used to identify unique variants gained or lost during drug exposure and mutant selection. Raw sequence data were also analyzed using TB-Profiler (version 3.1.12) to infer drug susceptibility profiles and to identify strain lineage (43, 44). The WGS data were deposited in the European Nucleotide Archive under accession number PRJEB55505.

Determination of growth rates. The growth rates were determined using the Bactec MGIT960 platform, as previously described (45). Briefly, cell suspensions with a turbidity equivalent to a McFarland 0.5 standard were created using actively growing cultures (day 21 to 28) of baseline and mutant strains from Middlebrook 7H10 plates containing OADC. Five hundred microliters of a 1:500 dilution of the cell suspension was used to inoculate MGIT tubes. The growth rate was determined as the time taken for cultures to grow from 5,000 to 10,000 GUs (replicates $n = 3$). A 2-way ANOVA was performed to determine whether there was any statistical difference between the baseline and mutant growth rates.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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