A CRISPR-guided mutagenic DNA polymerase strategy for the detection of antibiotic-resistant mutations in M. tuberculosis

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INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is the leading cause of death from infectious diseases. Most people with TB (drug-susceptible or drug-resistant) are cured after a medication regimen over 6–24 months.1,2 However, untimely treatment interruption or inappropriate use of antimicrobial drugs can result in drug resistance and treatment failure.3,4

A sharp increase in multidrug-resistant tuberculosis (MDR-TB) threatens human health. Spontaneous mutation in essential gene confers an ability of Mycobacterium tuberculosis resistance to anti-TB drugs. However, conventional laboratory strategies for identification and prediction of the mutations in this slowly growing species remain challenging. Here, by combining XCas9 nickase and the error-prone DNA polymerase A from M. tuberculosis, we constructed a CRISPR-guided DNA polymerase system, CAMPER, for effective site-directed mutagenesis of drug-target genes in mycobacteria. CAMPER was able to generate mutagenesis of all nucleotides at user-defined loci, and its bidirectional mutagenesis at nick sites allowed editing windows with lengths up to 80 nucleotides. Mutagenesis of drug-targeted genes in Mycobacterium smegmatis and M. tuberculosis with this system significantly increased the fraction of the antibiotic-resistant bacterial population to a level approximately 60–120-fold higher than that in unedited cells. Moreover, this strategy could facilitate the discovery of the mutation conferring antibiotic resistance and enable a rapid verification of the growth phenotype-mutation genotype association. Our data demonstrate that CAMPER facilitates targeted mutagenesis of genomic loci and thus may be useful for broad functions such as resistance prediction and development of novel TB therapies.
drug-resistance-related mutations. Continuous culture has been applied for the identification and prediction of drug-resistance mutations and is an effective way to isolate target mutants under antibiotic selection pressure.\(^1\)\(^1\)\(^2\)\(^\text{13}\)\(^\text{14}\) However, the mutation genotypes are very limited with this method because the spontaneous mutation rate in \textit{M. tuberculosis} is extremely low (2 \times 10^{-10}).\(^1\)\(^2\)\(^\text{15}\) Another strategy, randomized mutagenesis, is mainly based on error-prone PCR and requires complicated procedures, such as deletion of the chromosomal target gene and replacement with the mutated pool through homologous recombination (HR).\(^1\)\(^3\) Several genetic engineering strategies have been modified to improve the efficiency of HR in \textit{M. tuberculosis}, such as using a suicide vector for one-step or two-step allelic exchange.\(^1\)\(^4\) However, these methods are limited by the highly activated illegitimate recombination pathway in \textit{M. tuberculosis},\(^1\)\(^5\) the inefficient HR pathway,\(^1\)\(^6\) and the slow growth of the bacterium, such that 3–6 months is usually required to obtain a single recombinant mutant.\(^1\)\(^4\) Hence, developing advanced strategies to rapidly identify resistance-associated mutations is becoming increasingly important to support the development of appropriate TB treatment strategies and diagnostic tools.

CRISPR-guided DNA polymerase has the potential to facilitate targeted diversification of genes of interest in \textit{Escherichia coli}\(^1\)\(^7\)\(^\text{18}\) and Saccharomyces cerevisiae.\(^1\)\(^7\)\(^\text{18}\) This approach incorporates expression of a single guide RNA (sgRNA) targeting the gene of interest in bacteria harboring Cas9 nickase (nCas9)-DNA polymerase (PolA) fusion protein. The Cas9 nickase first recognizes and cleaves the target site of a specific gene. The gap generated by the nickase binds DNA polymerase and initiates nick translation. The formation of a nick allows the DNA polymerase to repair mismatches on both the leading and lagging strands.\(^1\)\(^9\) If the nick translation is finished by error-prone DNA polymerase, more substitution mutations are introduced into the new DNA fragment, resulting in random substitution mutations in the target region.\(^1\)\(^2\)\(^\text{10}\) Therefore, the combination of an sgRNA-guided Cas9 nickase and error-prone DNA polymerase can allow site-directed mutagenesis of target genes \textit{in vivo}. Unlike \textit{E. coli}, \textit{M. tuberculosis} lacks plasmids.\(^2\)\(^\text{0}\) It has been convincingly shown by many groups that \textit{M. tuberculosis} clinical isolates become resistant through point mutations in chromosomal genes and that these mutations arise spontaneously at low frequency.\(^2\)\(^\text{1}\) Given these features of \textit{M. tuberculosis}, it seems that this CRISPR-guided mutagenic DNA polymerase strategy may appropriately perform targeted mutagenesis for the identification of drug-resistant mutants in \textit{M. tuberculosis}.

In the current study, we used a CRISPR-guided DNA polymerase system in both fast-growing \textit{Mycobacterium smegmatis} and slow-growing \textit{M. tuberculosis}. Through protein structure alignment, we used DNA polymerase A from \textit{M. tuberculosis} to generate an error-prone DNA polymerase. By combining the highly compatible XCas9 nickase, a Cas9 variant recognizing NGN as the protospacer adjacent motif (PAM),\(^2\)\(^\text{1}\) with the error-prone DNA polymerase A from \textit{M. tuberculosis}, the CAMPER system was able to introduce random substitution mutations within an 80-bp-long editing window. Moreover, CAMPER not only efficiently induced site-directed mutagenesis in \textit{M. smegmatis} and \textit{M. tuberculosis} but also enabled the detection of the novel resistant mutants that likely involve inhibitor binding regions of a drug-targeted gene. Finally, when combined with high-throughput sequencing, the system successfully facilitated verification of the growth phenotype-mutation genotype association.

**RESULTS**

**Construction of a CRISPR-guided DNA polymerase system in mycobacteria**

With inspiration from the method previously reported by Halperin et al.,\(^1\)\(^7\) we aimed at constructing a CRISPR-guided error-prone DNA polymerase system in mycobacteria for random substitution mutations in a target gene. First, we used \textit{M. tuberculosis} DNA polymerase A (PolA) to generate error-prone DNA polymerase. The PolA possesses two functional domains: a flap endonuclease domain and a DNA polymerase domain.\(^2\)\(^\text{3}\) Point mutations in the large fragment of DNA polymerase A, the functional domain responsible for polymerization and proofreading, have been established to decrease the fidelity of the enzyme.\(^2\)\(^\text{3}\)\(^\text{2}\)\(^\text{4}\)\(^\text{5}\) For example, D424A at the metal ion site B of the 3’-5’ exonuclease domain inactivates the proofreading function of \textit{E. coli} PolA and increases the global mutation rate.\(^2\)\(^\text{4}\)\(^\text{2}\)\(^\text{6}\) The combination of I709F with D424A, a mutation inactivating the 3’-5’ exonuclease activity of \textit{E. coli} PolA, results in a 400-fold higher mutation rate than that of the wild type. In addition, the A759R substitution mutation increases the error rate of the polymerase in \textit{E. coli}.\(^2\)\(^\text{7}\)\(^\text{–}\)\(^\text{3}\)\(^\text{1}\) To determine the protein structure of \textit{M. tuberculosis} PolA, we performed structure modeling with \textit{M. smegmatis} PolA (PDB: 6VDE) as a structural template. Through protein structure alignment, we observed a similar structure of the DNA polymerase domain between \textit{E. coli} PolA and \textit{M. tuberculosis} PolA (Figure S1A). Of note, the positions of D424, I709, and A759 in \textit{E. coli} PolA corresponded to D405, I684, and A734 in \textit{M. tuberculosis} PolA, and these amino acids were conserved in \textit{E. coli} PolA and \textit{M. tuberculosis} PolA (Figure S1B), suggesting that D405, I684, and A734 in \textit{M. tuberculosis} PolA were critical for DNA synthesis. We therefore constructed PolA3M, a \textit{M. tuberculosis} PolA variant harboring D405A, I684N, and A734R substitution mutations, to be combined with Cas9 nickase. Since PolA has the activities of 5’-3’ exonuclease and 3’-5’ polymerase, the error-prone DNA polymerase A, after nicking by nCas9, may mutate the DNA fragment at the 5’ end of the nick through nick translation (Figure 1A).

We next designed a platform for gene editing containing an integrated plasmid for the expression of codon-optimized nCas9-PolA3M and a plasmid to express the sgRNA for guiding the system and TetR for regulation (Figure S2 and Table S1). The expression of both nCas9-PolA3M and sgRNA was under the control of an anhydrotetracycline (ATc)-inducible promoter. The editing efficiency of the tool was evaluated by measuring repair of a plasmid-encoded bleomycin resistance marker disabled by an early stop codon. For the system constructed by Cas9 cutting double-stranded DNA, the mutation frequency (7.81 \times 10^{-7}) was almost the same as that of the off-target control. However, for the system constructed with Cas9 nickase, the ratio of BleoR colony-forming units (CFUs)/viable...
CFUs for target editing \((2.44 \times 10^5)\) was 32.6-fold higher than that of the off-target control \((7.48 \times 10^5)\) on average (Figure 1B). We also applied this system to target the chromosomal locus of \(rpsL\), an essential gene that encodes the S12 ribosomal protein and is the primary site of streptomycin resistance mutations.32 The K88R mutation of \(RpsL\) is associated with high resistance to streptomycin,33 and an sgRNA targeting this region was designed. The mutant frequency calculations showed that the mutation rate of target editing on K88 \((5.98 \times 10^6)\) was 20.66-fold higher than that of the off-target control \((2.89 \times 10^7)\) (Figure 1C). The results strongly suggested that this
strategy effectively repaired the stop codon and led to the substitution mutation of the target gene.

Cas9 from *Streptococcus pyogenes* recognizes NGG as a PAM for cleavage activity, thus limiting the target site recognition of the system. To eliminate this constraint, we changed the Cas9 nickase to xCas9 nickase, a variant of Cas9 that carries point mutations at the PAM-identification domain and recognizes NGN as a PAM site.\(^7\) We further verified the editing efficiency for the system constructed from two types of XCas9 nickase, XCas9 (H840A) and XCas9 (D10A), which cleave different DNA strands at the same recognition site.\(^34\) We found that the editing efficiency was \(3.70 \times 10^{-5}\) for nXCas9(H840A)-PolA3M and \(4.32 \times 10^{-5}\) for nXCas9(D10A)-PolA3M, respectively 49.45-fold and 57.82-fold higher than that of the off-target control (Figure 1D). This result demonstrated that the system, which we called Cas9 and Mutagenic Polymerase for Evolving Resistance (CAMPER), constructed by XCas9 nickase, effectively mutated the target gene.

To assess the impact of ATc concentration on nXCas9-PolA3MTB expression, *M. smegmatis* cells expressing nXCas9-PolA3MTB and off-target sgRNA were treated with a range of ATc concentrations, and the expression of nXCas9 in *M. smegmatis* was examined by quantitative real-time PCR (qRT-PCR). Under induction with 200 ng/mL ATc, the nXCas9-PolA3MTB achieved maximal induction, to an expression level 43.45-fold higher than that of the control without ATc induction (Figure 1E). We also found that the maximal expression of nXCas9-PolA3MTB persisted in the cells for approximately 24 h but decreased dramatically after 48 h (Figure 1F). Importantly, maintaining maximal induction of nXCas9-PolA3MTB resulted in slight growth defects (Figures 1G–I), but no significant change on bacterial morphology was observed (Figure 1I), thus suggesting slight proteotoxicity for expression of nXCas9-PolA3MTB fusion protein in mycobacteria.

Subsequently, the targeted bleo\(^6\) gene was analyzed via high-throughput targeted amplicon sequencing (Figure 2A). We found an increase in mutagenesis within a 50-nt window at the 5' side of the nick and interestingly, a relatively low elevation in mutation frequency within a 10- to 15-bp window ~20 bp from the nick site in the opposite direction. No such increase was observed in cells expressing off-target sgRNA and nXCas9-PolA3MTB (Figure 2B). This result suggested that the fusion protein nXCas9-PolA3MTB advanced along the DNA strand in both 5'-3' and 3'-5' directions from the nick. To confirm this feature, we used the system to repair two early stop codons located at different sides of the nick in bleo\(^6\). For the plasmid with a stop codon in the 5' window of the nick, the mutation frequency for target editing (3.6 \(\times\) \(10^{-4}\)) increased by 100-fold over that of the off-target control (3.67 \(\times\) \(10^{-5}\)), whereas the mutation frequency for the plasmid harboring a stop codon at the opposite side of the nick was 9.8 \(\times\) \(10^{-5}\) (Figure 2C). The genotypes for repaired clones exhibiting bleomycin resistance were confirmed by Sanger sequencing (Table S3). The results indicated that the CRISPR-guided system edited target fragments at both sides of the nick. Importantly, this system also displayed a double-direction editing feature when heterogeneous expression of enCas9-PolA3MTB occurred in *E. coli*, and its editing efficiency appeared to be higher than that of EvolvR\(^23\) (Figure 2D). In addition, although the target site is a GC-rich region, we observed that two other nucleotides were mutated (Figure 2E). It appeared that CAMPER was more likely to mutate G/C into A/T, rendering it more applicable in G+C-rich bacteria such as *Mycobacterium*.

Off-target effects of nXCas9-PolA3MTB are of great concern. A previous study has shown that off-target effects of Cas9 can occur at sites with five to six mismatches out of 20 bp sequence relative to the protospacer sequence of sgRNA.\(^25\) To evaluate the off-target ratio, we amplified potential target sites with four mismatches relative to the sgRNA targeting site of bleo\(^6\) from ten bleo\(^6\) isolates expressing nXCas9 and bleo\(^6\)-specific sgRNA. Using Sanger sequencing, we observed no difference between the wild-type sequence and these sequences (Table S4), in agreement with previous findings.\(^21\) It seems likely that CAMPER did not reveal significant off-target effects.

**Testing CAMPER in *M. smegmatis* and *M. tuberculosis***

Next, we tested CAMPER for its mutagenesis editing ability in *M. smegmatis* through several types of mutant frequency calculation assay. We first evaluated the mutagenesis editing on a plasmid gene. A plasmid for expression of nXCas9-PolA3MTB and another plasmid for expression of bleo\(^6\) disabled by an early stop codon as well as an sgRNA targeting the early stop codon were transformed into *M. smegmatis*. Different volumes of electroporation cultures were subcultured into 1 mL of medium and cultivated until saturation. For the groups with a 100-µL subculture volume, the value of Bleo\(^6\)-CFUs/viable CFUs was 2.83 \(\times\) \(10^{-5}\), which was 118-fold higher than that of the off-target control (2.41 \(\times\) \(10^{-7}\)). No significant difference was observed in the mutation frequency among groups with different subculture volumes (Figure 3A). These results indicated that CAMPER could efficiently edit plasmid genes. To test its editing efficiency on chromosomal genes, we decided to target the rpoB gene, which encodes the RNA polymerase β subunit whose mutations confer resistance to rifampicin.\(^36\) Via an sgRNA targeting rpoB, the target-editing strain displayed a 91-fold higher mutation rate (1.31 \(\times\) \(10^{-5}\)) than that of the off-target control strain (1.44 \(\times\) \(10^{-6}\)) with a 100-µL subculture volume, and no significant differences were observed in the mutation rates among groups with different subculture volumes (Figure 3B).

Inspired by the success of CAMPER application in *M. smegmatis*, we next explored its programmability for genome editing in *M. tuberculosis*. To simplify the procedure in the slowly growing species, we generated an optimized system without TetR. In agreement with the observations in *M. smegmatis*, constitutive expression of nXCas9-PolA3M with an sgRNA targeting K88 of *M. tuberculosis* appeared that CAMPER was more likely to mutate G/C into A/T. This feature, we used the system to repair two early stop codons located at different sides of the nick in bleo\(^6\). For the plasmid with a stop codon in the 5' window of the nick, the mutation frequency for target editing (3.6 \(\times\) \(10^{-4}\)) increased by 100-fold over that of the off-target control (3.67 \(\times\) \(10^{-5}\)), whereas the mutation frequency for the plasmid harboring a stop codon at the opposite side of the nick was 9.8 \(\times\) \(10^{-5}\) (Figure 2C). The genotypes for repaired clones exhibiting bleomycin resistance were confirmed by Sanger sequencing (Table S3). The results indicated that the CRISPR-guided system edited target fragments at both sides of the nick. Importantly,
mutations confer resistance to bedaquiline, the percentage of bedaquiline-resistant population ($4.83 \times 10^{-5}$, Figure 3E) was 22.31-fold higher than that of the off-target control ($2.17 \times 10^{-6}$). More importantly, consistent with the observation in M. smegmatis and M. tuberculosis H37Ra, the system also exhibited a capacity for efficient genome editing in M. tuberculosis H37Rv (Figures 3F and 3G).

All of the above results demonstrated that the CRISPR-guided DNA polymerase system effectively edited a targeted chromosomal gene in both M. smegmatis and M. tuberculosis.

### Rapid discovery of novel drug-resistant mutations via CAMPER

Elucidating the interaction between drug-resistant mutations and antimicrobial drugs may facilitate the design of novel antibiotics before their clinical applications. We wondered whether our strategy might facilitate the screening of drug-resistant mutations. To this end, we chose AtpE, the target protein of the latest anti-TB drug bedaquiline, as a proof of principle. Bedaquiline inhibits bacterial ATP synthase by targeting the c subunit of the mycobacterial F-ATP synthase AtpE37–39 (Figure 4A), which is used to treat MDR-TB along with other anti-TB drugs and represents the first novel class of anti-TB agents in more than 40 years.40
To detect the drug-resistant mutations, we designed six sgRNAs to cover the 261-bp \textit{atpE} coding sequence (Figure 4B). After culture saturation, bacteria cells were challenged by growth on agar plates containing various concentrations of bedaquiline. We observed that the strains harboring the off-target control, sgRNA-13R, sgRNA-39R, or sgRNA-153R, did not grow under bedaquiline selection, even at the lowest concentration (Figure 4B). For the strains with sgRNA-215F targeting the E65-N71 region of AtpE (c subunit), which has been demonstrated to interact with bedaquiline,\textsuperscript{41} the resistance level was highest (Figure 4C). A recent study has demonstrated that the occurrence of a mutation at I66 of AtpE is associated with a 4- to 64-fold bedaquiline minimum inhibitory concentration (MIC) increase in clinical \textit{M. tuberculosis} isolates,\textsuperscript{42,43} corresponding to I70 in \textit{M. smegmatis}. To further confirm the resistance phenotype response to genotype, we verified mutation type I70M from ten clones isolated from bedaquiline-resistant strains harboring sgRNA-215F. As expected, the mutation of I70M conferred bedaquiline resistance (Figure S3).

We further used the capacity of CAMPER to diversify the rifampicin resistance determining region (RRDR) of \textit{rpoB} gene to verify the mutation that confers rifampicin resistance in \textit{M. smegmatis} (Figure 4D). In total, 31 sgRNAs were designed to cover the coding region, and the saturated cultures were plated on agar plates containing rifampicin (Figure 4E). The percentage of rifampicin-resistant mutants per viable CFU was calculated. The strains carrying sgRNA-3, sgRNA-12, and sgRNA-16 displayed higher mutation frequencies than that of the remainder, while the expression of sgRNA-11 resulted in the highest mutation frequency, which was 97.43-fold higher than that of the off-target. The corresponding regions targeted by the above sgRNAs were RRDR-I, RRDR-III, and RRDR-I, respectively. These results are consistent with the report from previous research that the resistance toward rifampicin was related to the spontaneous mutations within five RRDRs in \textit{RpoB} containing the N-terminal cluster and clusters I, II, and III, with the vast majority of mutations being concentrated in cluster I (RRDR-I)\textsuperscript{36} (Figure 4F).
Collectively, this strategy should be useful for rapidly discovering drug-resistant mutations.

**Using CAMPER to rapidly analyze the growth phenotype-mutation genotype association**

Drug-resistance mutations are often associated with a fitness cost as compared with the antibiotic susceptibility of bacteria in the absence of antibiotic selection.\(^{44,45}\) Prediction of future drug-resistant TB epidemics depends on the fitness cost of drug-resistance mutations.\(^ {46,47}\) Identification of the fitness of mutation genotype that occurs for essential genes is of great concern in mycobacteria. We assumed that, combined with high-throughput sequencing, our strategy might contribute to rapid analysis of the correlation between growth phenotype and mutation genotype. We chose the RRDR region of *M. smegmatis rpoB* as a proof of concept, because the amino acid sequence of the RRDR is highly conserved between *M. tuberculosis* and *M. smegmatis*. It is well known that rifampicin resistance in *M. tuberculosis* conferring mutation in the RRDR imposes a fitness cost when grown in the absence of rifampicin.\(^ {48}\)

Using CAMPER, we developed a framework to rapidly evolve *rpoB* and determine the fitness of its variants (Figure 5A). The sgRNAs with high editing efficiency were chosen for library construction (Figure S4). Subsequently, we screened out the rifampicin-resistant
Figure 5. Using CAMPER to rapidly evolve rpoB RRDR of M. smegmatis

(A) Schematic illustration of the competitive assay workflow for determining the fitness landscape of RRDR. pCAMPER and pGuider were both transformed into M. smegmatis. With the expression of gRNAs for target editing, CAMPER performed random substitution mutation among the 81-bp loci of RRDR. During cultivation, the system mutated the target region continuously, owing to induction with ATc. Mutagenesis accumulated among the entire viable population. After the saturation stage, all cultures were plated on agar containing rifampicin to screen out rifampicin-resistant (RIFR) mutants, and were considered as the T0 library. For competitive assays, equal amounts of RIFR mutants were subcultured in 7H9 medium containing rifampicin, and cultures without rifampicin were used as a control. Samples were collected at T1, and the 81-bp DNA fragment of RRDR was amplified via PCR. Genotypes and related frequencies were further verified via Illumina HiSeq sequencing. The fitness of each genotype was evaluated as the increase in frequency under antibiotic selection relative to that of wild-type RRDR. (B) The number of genotypes changed under treatment with different concentrations of rifampicin. (C) Surface representation of the M. smegmatis RNA polymerase β subunit and steric hindrance of rifampicin binding to the RRDR. The RRDR is in blue, and the region with mutations detected at T0 is in pink. (D) Fitness landscape of RRDR under treatment with rifampicin at 0, 80, or 160 μg/mL at T1. Each tile
mutants by plating the pool on Luria-Bertani (LB) agar plates containing rifampicin. After incubation, all colonies were collected from the resistant plate for in vitro competitive experiments, and this point time was considered time zero (T0). Competitive experiments were performed under three concentrations of rifampicin (0, 80, and 160 μg/mL). The indicated samples were collected in log phase (Figure S5A). To accurately estimate the fitness of the variants, we included 41 non-synonymous mutation genotypes with frequencies ≥0.06% at T0 in further analysis, discarding multiple mutations, indels, or deletions (Figure 4B and Table S5). The genotype frequencies were highly correlated between biological replicates under treatment with rifampicin at 0, 80, and 160 μg/mL, and the mean R of all replicate pairs was 0.9999 (Figure S3B). In addition, we also verified ten mutation genotypes directly isolated from the T0 rifampicin-resistant strains (RiP®) pool through Sanger sequencing (Table S6). Among these mutations, nine were identical to the mutation genotypes identified from the T0 library, one of which carried three point mutations, and the serine at position 522 was substituted with valine, which is an RIFR-related mutation that has not been reported in clinical M. tuberculosis isolates to date.

The number of mutation genotypes decreased to 24 under the condition without antibiotics, and this number increased to 38 under treatment with 80 μg/mL rifampicin and to 36 under selection with 160 μg/mL rifampicin (Figure 5B). A total of 74.1% (20/27) of the amino acid mutations in RRDR were represented in the T0 library, one of which carried three point mutations, and the serine at position 522 was substituted with valine, which is an RIFR-related mutation that has not been reported in clinical M. tuberculosis isolates to date.

On the basis of these data, we conclude that CAMPER combined with high-throughput sequencing enables rapid determination of the relationship between growth phenotype and mutation genotype in mycobacteria.

**DISCUSSION**

In this study, we developed an approach for mutagenesis of targeted genomic loci in mycobacteria by using a CRISPR-guided DNA polymerase system. This system may significantly enable TB research, particularly on the evolution of antimicrobial resistance and anti-TB drug development. First, this approach enables targeted diversification of all nucleotides at user-defined loci in M. tuberculosis within 2 months. Moreover, whereas conventional approaches rely on laborious genomic engineering procedures, diversification of the genomic region edited by our system requires only co-expression of nXCas9-PolA3MTB and a 20-nt sgRNA, thus making this system efficient in slowly growing mycobacteria. Second, CAMPER can be useful for understanding protein-drug interactions. As demonstrated by our data, this system can be used to rapidly identify the drug-resistant mutations of gene responses to its protein function. Finally, as demonstrated in this study, predictions of drug-resistance mutations in mycobacteria according to our framework may have different potentials for clinical applications. For example, this framework could be used to perform a rapid preliminary evaluation of whether novel mutations are likely to confer the ability to resist therapy, thus contributing to genotype-phenotype predictions in diagnostics. In addition, this framework could be used to map novel variants, which may emerge after deployment in clinical practice, for assessment of novel agents.

Two caveats in our study should be discussed. First, we observed that high expression of nxCas9-PolA3MTB slightly retarded bacterial growth, suggesting that some degree of proteotoxicity may result from nxCas9-PolA3MTB expression. In fact, first-generation Cas9py proteotoxicity has been reported in mycobacteria. Although second-generation Cas9nt slices exhibits low toxicity and robust utility in gene knockdown and knockout in mycobacteria, Cas9nt is unable to target certain specific sequences because of the limitation of its unique PAM sequences. Therefore, exploring novel Cas9 with low toxicity and high flexibility in mycobacteria would enable further improvements in the performance of this tool. Second, our data demonstrated that the editing window of nxCas9-PolA3MTB was ~80 bp. Also, the mutation frequency relies on the flexibility of the DNA polymerase domain of PolA. Rationally designed mutation of the DNA polymerase domain to increase the mutation frequency and window length will be highly important in future research.

Interestingly, we observed that CAMPER has an extended mutagenesis window length. This phenomenon in mycobacteria was not
previously observed in *E. coli*, in which mutagenesis induced by *E. coli* PolA only occurs at 3′ of the nick sites. CAMPER also displayed a double-direction editing feature with heterogeneous expression of enCas9-PolA3MTB in *E. coli*, indicating that a difference in structure between PolA<sup>TB</sup> and *E. coli* PolA, rather than in genetic backgrounds (*E. coli* versus mycobacteria) may result in this editing feature. Although recent work<sup>11</sup> has reported a similar protein structure between *E. coli* PolA and *M. smegmatis* PolA, the 3′-5′ exonuclease subdomain of *M. smegmatis* PolA lacks several carboxylate-containing amino acids that interact with essential metal ions at the 3′-5′ exonuclease active site of the *E. coli* PolA Klenow large fragment.<sup>24</sup> This distinctive feature results in a vestigial 3′-5′ exonuclease and a lack of proofreading activity in *M. smegmatis* PolA. In addition, the vestigial 3′-5′ exonuclease subdomain of *M. smegmatis* PolA contains a unique single-manganese ion binding site, which cannot engage with other metal ions and is not conserved in *E. coli* PolA.<sup>25</sup> These findings may to some extent contribute to the action of *M. tuberculosis* PolA observed herein, because the interaction with metal ions is essential for the activity of 3′-5′ exonuclease and its binding to single-stranded DNA. However, the mechanism underlying the bidirectional nick translation activity of PolA3MTB remains to be elucidated.

MATERIALS AND METHODS

**Strains and media**

*M. tuberculosis* H37Ra and H37Rv were cultured at 37°C in Difco Middlebrook 7H9 broth or 7H10 plates supplemented with 0.2% glycerol (7H9) or 0.5% glycerol (7H10), 0.05% Tween 80, and OADC (oleic acid-albumin-dextrose-catalase). *M. smegmatis* MC<sup>2</sup> 155 was grown at 37°C in LB agar plates, or 7H9 broth supplemented with 0.05% Tween 80 and 10% oleic acid ADC. When indicated, antibiotics and inducers were used at the following concentrations: kanamycin (25 μg/mL), hygromycin (100 μg/mL), bleomycin (25 μg/mL), streptomycin (25 μg/mL), rifampicin (80 μg/mL), bedaquiline (0.0125 μg/mL), and ATc (200 ng/mL). The *E. coli* strain DH5α was used for plasmid cloning and was cultivated at 37°C in LB broth or agar plates. When indicated, antibiotics were used at the following concentrations: kanamycin (50 μg/mL), hygromycin (150 μg/mL), and streptomycin (50 μg/mL).

**Plasmid construction**

All plasmids (Table S1) were constructed with a one-step cloning strategy. The integrating vector pCAMPER consisted of a kanamycin resistance cassette, integrase from phage L5, a high-copy-number origin of replication, and genes encoding nXCas9-PolA3MTB under control of the uv1Steto promoter. To allow for simple cloning of sgRNA, we constructed a replicative vector, pGuider, derived from pRH2521. In brief, an sgFPP cassette under the control of the J23115 promoter was cloned into the BbsI-digested pRH2521. The pGuider2 was identical to pGuider except that it contained a bleo<sup>R</sup> gene carrying an E77<sup>+</sup> mutation. The pGuider3 was identical to pGuider except that it contained a bleo<sup>R</sup> gene carrying a W23<sup>+</sup> mutation. The full plasmid sequences are provided in Table S2. Details on plasmid construction are available upon request.

mRNA quantification

To identify the optimal ATc concentration for nXCas9-PolA3MTB expression induction, we grew *M. smegmatis* harboring pCAMPER and pGuider to log phase and performed induction with ATc at 0, 10, 50, or 200 μg/mL, respectively. After cultivation at 37°C for 48 h, we collected 10 mL of culture for each sample, and three replicates were used for each condition. To further verify the steadiness of the fusion protein expression in *M. smegmatis*, *M. smegmatis* harboring pCAMPER and pGuider for an off-target control was grown to log phase. After induction with ATc at 200 ng/mL, 10 mL of culture was collected at 0, 8, 24, 48, and 96 h, respectively. The collected cultures were harvested by centrifugation. After removal of the supernatant, the cell pellet was resuspended in RNAeasy isolation reagent (Vazyme) and disrupted by bead beating with a TissueLyser II (Qiagen). Total RNA was precipitated by the addition of isopropanol and collected by centrifugation, the supernatant was discarded, and the mRNA pellet was washed with 75% ethanol. After drying of the pellet, mRNA was dissolved in RNase-free H2O. The contaminating genomic DNA was digested with gDNA wipeMix (Vazyme). The cDNA was prepared from purified mRNA with HiScriptII qRT SuperMix II (Vazyme) through reverse transcription. The cDNA levels of target genes were then quantified by qRT-PCR on a CFX96 cycler (Bio-Rad) with AceQ Universal SYBR qPCR Master Mix (Vazyme) as a dye for fluorescence signal detection. All qPCR primers were determined to be >95% efficient, and the cDNA masses tested were experimentally validated to be within the linear dynamic range of the assay. Signals were normalized to those of the housekeeping sigA transcript and quantified with the ΔΔCt method. Error bars were 95% confidence intervals of the three technical replicates.

**Gene editing in *M. smegmatis* and *M. tuberculosis***

For *M. smegmatis*, a total of 100 ng of pGuider was transformed into *M. smegmatis* harboring pCAMPER electroporation-competent cells. After 2 h of recovery at 37°C, unless otherwise stated, 100 μL of culture was inoculated into 1 mL of 7H9 medium containing kanamycin, hygromycin, and ATc. The cultures were cultivated at 37°C until reaching the saturation stage, and 100 μL of culture was plated on an LB agar plate containing corresponding antibiotic for target mutant screening. For counting of viable CFUs, 100 μL of 1:10<sup>6</sup> diluted culture was plated on an LB agar plate containing kanamycin. After incubation at 37°C for 72 h, resistant CFUs and viable CFUs were counted, and the mutation frequencies were verified according to the values of resistant CFUs/viable CFUs. Off-target gRNA was transformed as a negative control.

For *M. tuberculosis*, a total of 100 ng of pGuider for expression of sgRNA targeting the 3′-terminal regions of rpoB RRDR, K88 of rpsL, or I66 of atpE was transformed into *M. tuberculosis* harboring pCAMPER electroporation-competent cells. For both *M. tuberculosis* H37Ra and H37Rv, 100-ml logarithmic phase cultures of were collected by centrifugation (5,000 × g, 10 min) and washed three times with gradually decreasing volumes (40, 20, and 10 mL) of 10% glycerol at room temperature, and finally resuspended in 1 mL of 10% glycerol. For high yield of transformants, 100 ng of plasmid
was mixed thoroughly with 200 μL of fresh electrocompetent bacilli, which were then transferred to a chilled 0.2-cm electrode gap GenePulser electroporation cuvette (Bio-Rad) and subjected to electroporation using a GenePulser Cxell electroporator (Bio-Rad) at the following settings: 2.5 kV, 1,000 Ω, and 25 μF. After 24 h of recovery at 37°C, 100 μL of culture was then inoculated into 1 mL of 7H9 medium containing kanamycin, hygromycin, and ATc. The cultures were cultivated at 37°C until saturation stage, and 100 μL of culture was plated on a 7H10 agar plate containing rifampicin, streptomycin, or bedaquiline for target mutant screening. For viable CFU counting, 100 μL of 1:10^6 diluted culture was plated on a 7H10 agar plate containing kanamycin. After incubation at 37°C for 28 days, resistant CFUs and viable CFUs were counted, and the mutation frequencies were verified by the values of resistant CFUs/viable CFUs.

**Sample preparation for high-throughput sequencing**

For NovaSeq next-generation sequencing, genomic DNA was extracted from samples of interest with a HiPure Bacterial DNA kit (Magen). The related oligonucleotides were used for the amplification of the target region in a 20-cycle PCR reaction with high-fidelity Phanta DNA polymerase (Vazyme), with 200 ng of genomic DNA as the template. The PCR products were then purified with FastPure DNA Mini Columns (Vazyme). For the second PCR reactions with 100 ng of the first-round PCR product as the template, the gene of interest was amplified with oligonucleotides containing Illumina sequencing adapters, an index for sample separation and primers for sequencing with ten thermocycles. The well-prepared samples were submitted to the Novogene Sequencing Center & Clinical Lab (Tianjin, China) for quality control and sequencing. The samples were submitted to the Novogene Sequencing Center & Clinical Lab (Tianjin, China) for quality control and sequencing. The pooled sample was run on an Illumina NovaSeq instrument by means of a 150-bp paired-end read NovaSeq Reagent Kit. The resulting raw data were then converted into fastq format for splicing, filtration, and analysis.

**High-throughput sequencing data analysis**

The raw reads were filtered with fastp v.0.2 (https://doi.org/10.1093/bioinformatics/bty560) through the following procedures: (1) primers and adapter sequences were removed (default); (2) the first and last five nucleotides were trimmed (-f 5 -t 5); (3) reads with N bases (-n 0) or low-quality bases (-u 100) were removed; (4) paired-end reads whose R1 did not perfectly match part of the reverse complement R2 were removed (--overlap_diff_limit 0 –overlap_diff_percent_limit 0); and (5) each pair of reads were merged into a single read if they overlapped (-m), or were removed otherwise. Merged reads were mapped to the wild-type rpoB-RRDR gene (GenBank: ABK70312) or blecR gene (GenBank: CAA02067) to generate the alignment files via bwa v0.7 (https://doi.org/10.1093/bioinformatics/btp324) and samtools v0.1.18 (https://doi.org/10.1093/bioinformatics/btp352). Variant calling was performed with VarScan v.2.3.9 with parameters: –min-coverage 1 –min-reads2 1 –p-value 0.99 –variants 1 –min-var-freq 0 (https://doi.org/10.1093/bioinformatics/btp373). Because the highest frequency variant of the empty vector control was 0.06%, the variants in the experimental group were discarded from subsequent analysis if the frequency was below 0.06% (–min-var-freq 0.0006).

**Construction of an RRDR gene variant pool**

To construct a mutation library covering all loci of RRDR, we designed six sgRNAs for mutagenesis. Three targeted the 5’ terminus (101F, 113F, and 91R) and the remainder targeted the 3’ terminus (40R, 67F, and 74F). The pGuider plasmids for expression of either the six sgRNAs or off-target gRNA were transformed into *M. smegmatis* harboring pCAMPER electroporation-cell competent. For each condition, 100 μL of culture was inoculated into 1 mL of 7H9 medium containing kanamycin, hygromycin, and ATc. The cultures were cultivated at 37°C until reaching saturation stage. To verify the editing efficiency of the systems guided by different sgRNAs, we calculated the mutation frequencies with CFU assays: 100 μL of culture was plated on an LB agar plate containing rifampicin for target mutant screening. For viable CFU counting, 100 μL of 1:10^6 diluted culture was plated on an LB agar plate containing kanamycin. After incubation at 37°C for 72 h, resistant CFUs and viable CFUs were counted, and the mutation frequencies were verified according to the values of resistant CFUs/viable CFUs. Three replicates were used for each condition. All remaining cultures were prepared for competition assays.

**Competition assays**

The well-prepared RRDR mutant library was first plated on LB agar containing kanamycin and rifampicin to screen out rifampicin resistance-related isolates, then 100 μL of culture was plated on each plate. After incubation at 37°C for 72 h, all colonies were collected from the LB agar plate by washing with saline and were considered as the rifampicin-resistant strain T0 library. A 5-mL sample was then collected for genotype analysis. Three replicates were used. Aliquots (100 μL) of the pooled culture were subcultured in 100 mL of 7H9 medium containing rifampicin at 0, 80, or 160 μg/mL. Three biological repeats were examined for each condition. A total of 50 mL of sample culture was collected at the indicated time points, where OD600 was 0.6. At each time point, the OD600 was measured with a Nano-photometer (IMPLEN) and represented by the value of OD600.

**Estimation of the growth phenotype-mutation genotype association**

To determine the frequency of each barcode within a sample, we performed paired-end 150-bp sequencing on each sample on the Illumina NovaSeq platform. RRDR sequences of 81 nt were extracted from the sequencing reads, and the sequences with nonidentical sequences from matching forward and reverse sequencing reads were excluded from further analysis. To ensure accurate estimation of the fitness, we analyzed 41 genotypes with frequency ≥ 0.06% from the technical repeats at T0. The fitness at each time point of each competitive growth assay was calculated for individual mutations as f1(Mutant)/f0(Mutant), where f1(Mutant) is the frequency of sequencing reads of a certain genotype at T1 and f0(Mutant) is the frequency of sequencing reads of the same genotype at T0.
Strain construction
Deletion of rpoB was generated by transformation with a second copy of the gene and integration at the L5 phage integration sites, and subsequent mycobacterial recombineering as previously described to delete the endogenous copy. To confirm the phenotype of the rpoB mutants observed in competitive experiments, we constructed two variants of rpoB by one-step cloning. In brief, two adjacent mutated target fragments with 20–15 bp homologous sequences were amplified by PCR. The PCR products were verified by electrophoresis and purified with a gel extraction kit (Omega). The pGM CZ-rpoB-SmR plasmid was digested with FastDigest NsiI (Thermo Fisher) and FastDigest XhoI (Thermo Fisher) at 37°C for 1 h. Ligation was performed at a vector/insert ratio of 1:3 with a Seamless Cloning Kit (Beyotime, China). The product was transformed into DH5-α-resistant cells and selected on streptomycin (100 μg/mL). Recombinant plasmids were purified, and the corresponding rpoB variant was sequenced to confirm the mutation. The plasmid containing the rpoB mutation was transformed into M. smegmatis ΔrpoB attB:rpoB bleO competent cells. L5 swaps were performed as previously described.

Three-dimensional analysis of RpoB variant proteins
Three-dimensional structural data for M. smegmatis RpoB (PDB: 6CCV), ATP synthase (PDB: 7JG8), and E. coli PolA (PDB: 1KLN) with high resolution were downloaded from the PDB database. The architecture of full-length M. tuberculosis PolA was modeled with Swiss-Model. The PolA of M. smegmatis (PDB: 6VDE) served as a structural template. The protein structure was visualized with PyMOL software.

MIC assays
To determine the rifampicin susceptibility of the RRDR variants, we performed rifampicin MIC assays. In brief, single colonies of the target RRDR mutants were inoculated in 7H9 medium separately and cultivated overnight at 37°C. The cultures were then adjusted to an OD₆₀₀ of 0.5 with saline and diluted at a ratio of 1:10. Next, 20 μL of diluted culture was added into 180 μL of 7H9 medium containing rifampicin at 0, 4, 8, 16, 32, 64, or 256 μg/mL in a 96-well plate. A Nano-photometer (Implen) was used to measure the optical density of each well after 72 h of growth at 37°C. Three biological replicates of each RRDR mutant under each rifampicin concentration were verified, and a wild-type M. smegmatis MC² 155 strain was used for comparison.

Measurement of growth curve
Fresh single colonies of the target strains were inoculated in 7H9 medium separately and cultivated overnight at 37°C. The cultures were then adjusted to an OD₆₀₀ of 0.5 with saline, and diluted at a ratio of 1:10. Next, 20 μL of diluted culture was added to 180 μL of 7H9 medium in a 96-well plate. A Nano-photometer (Implen) was used to measure the optical density of each well every 3 h. Three replicates were used for each strain. The growth curve was plotted in Prism 8.

Statistical analysis
Statistical analysis was performed in Prism (version 8.0; GraphPad Software). Statistical significance of paired comparison data was assessed with Student’s paired t test. The statistical significance of data with multiple confounding factors was assessed with analysis of variance. A p value of 0.05 or lower was considered statistically significant.

DATA AND CODE AVAILABILITY
The data that support the findings of this study are available from the corresponding authors upon request. High-throughput sequencing data have been deposited as an NCBI BioProject under accession number GenBank: PRJNA716418. Also, the code that supports the findings of this study is available at https://github.com/shencong456/rpoB_bleO.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.07.004.

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AUTHOR CONTRIBUTIONS
S.F., L. Liang, and C.S. contributed equally to this study. G.-B.T. conceived the idea, and designed and supervised the study. L. Liang, S.F., D.L., and L. Liu conducted the experiments and produced the tables and figures. J.L. and W.L. searched the literature. C.S. contributed to data analysis and interpretation. S.F., L. Liang, and G.-B.T. wrote the manuscript, which was reviewed and edited by G.M.C. and Y.D. All authors reviewed, revised, and approved the final report.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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