

Interaction between *rpsL* and *gyrA* mutations affects the fitness and dual resistance of *Mycobacterium tuberculosis* clinical isolates against streptomycin and fluoroquinolones

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Background: The interaction between different drug-resistant mutations is important to the development of drug resistance and its evolution. In this study, we aimed to reveal the potential relationships between mutations conferring resistance to two important antituberculosis drugs streptomycin (STR) and fluoroquinolones (FLQ).

Materials and methods: We used an in vitro competitive fitness assay to reveal the interactions between different mutations of *rpsL* and *gyrA* in drug-resistant *Mycobacterium smegmatis*, followed by the analysis of the frequency of *rpsL* and *gyrA* mutation combinations in 213 STR–FLQ dual-resistant clinical *Mycobacterium tuberculosis* isolates from Sichuan region, which was also investigated by the whole genome data from 3,056 global clinical *M. tuberculosis* isolates.

Results: The strains with K43R and K88R mutation in *rpsL* showed no difference in relative fitness compared with their susceptible ancestor, while K43N, K43M, K43T, and K88E exhibited a significantly lower relative fitness ($P < 0.05$). For the FLQ-resistant mutants, all mutation types showed no difference in their relative fitness. Among STR–FLQ dual-resistant *M. smegmatis* strains, a lower fitness was detected in those with K43N/M/T and K88E instead of K43R and K88R mutations in *rpsL*. Among *M. tuberculosis* isolates harboring *rpsL* and *gyrA* dual mutations, the most two frequent combinatorial mutation types were K43R/D94G ($n = 37$) and K43R/A90V ($n = 24$), with the former being the most frequent one by both in vitro tests and clinical survey.

Conclusion: Our results suggest that the interaction between *rpsL* and *gyrA* mutations affects the fitness cost in STR–FLQ dual-resistant *M. smegmatis* and also the predilection of mutation combinations in clinical *M. tuberculosis* isolates.

Keywords: *Mycobacterium*, fitness, dual resistance, epistasis

Introduction

Tuberculosis (TB) remains one of the deadliest infectious diseases around the world. According to the World Health Organization, an estimated 10.4 million people developed TB, 1.4 million died from the disease in 2015, and an additional 0.4 million deaths resulted from TB disease among people living with HIV.¹ The emergence and spread of multidrug-resistant TB (MDR-TB) with 3.3% of newly diagnosed TB cases worldwide, which was associated with poor treatment outcomes, aggravated the burden of TB treatment in many regions of the world.² When it came to previously treated TB cases, 20% were identified as MDR-TB.¹ However, the understanding of the interaction among various drug resistances in TB is quite poor.

Epistasis refers to the phenomenon of phenotypic effect exerted by one gene (locus) over another leading to the fact that epistatic mutations have different effects in combination than individually.³ Acting as the primary factor in molecular evolution, epistasis has been revealed in many processes.⁴ Recently, epistasis was reported as an important evolutionary force for the emergence of multidrug-resistant bacteria.^{5,6} In mycobacteria, resistance to rifampicin (RIF) and ofloxacin was reported to be affected by epistasis, in which different combinations of mutations in drug-resistant genes resulted in the varying degree of loss in fitness.⁷ However, a more generalized epistatic interaction in mutations conferring resistance to other anti-TB drugs remains unknown.

Streptomycin (STR) was first applied in clinical treatment for TB in 1946,⁸ although its use was reduced gradually in western countries due to its strong side effects and emergence of drug resistance. Nonetheless, in most developing countries, STR has been used as one of the first-line anti-TB drugs up to date, which induced serious STR resistance in these countries.⁹ Fluoroquinolones (FLQ), as an important second-line anti-TB drug, are critical to the treatment of MDR-TB.¹⁰ With the development of FLQ resistance, MDR-TB may progressively lead to the emergence of extensively drug-resistant (XDR), even totally drug-resistant (TDR) TB.² During the treatment of MDR-TB cases, STR is usually prescribed in combination with FLQ because of the lack of therapeutic activity of isoniazid and RIF against MDR-TB. However, little is known about the interactive effects between STR- and FLQ-resistant mutations in mycobacteria.

In this study, with the aim to explore the interaction between STR- and FLQ-resistant mutations, *Mycobacterium smegmatis* was used as a model to investigate the epistasis between mutations conferring resistance to STR and FLQ and the data obtained were then compared with the frequency of STR–FLQ dual-resistant gene mutations in clinical *Mycobacterium tuberculosis* isolates.

Materials and methods

Bacterial strains and culture conditions

The wild-type *M. smegmatis* strain mc²155 was used as the starting strain for the screening of drug-resistant strains and the reference for the competitive fitness experiments. The mycobacteria were cultured in Middlebrook 7H9 broth supplemented with 0.05% Tween 80 (7H9-Tw). Bacteria were cultured at 37°C with shaking at 180 rpm.

Microplate-based growth assay for minimal inhibitory concentration (MIC) determination

MIC determination was accomplished as previously described with minor modifications.¹¹ *M. smegmatis* was shaken at 37°C in 7H9-Tw till reaching optical density (OD₆₀₀) ~0.8. The cells were spun down, washed twice with 7H9-Tw, and diluted by 100-fold in 7H9-Tw. A total of 200 µL of each culture was placed in a 96-well clear bottom plate. After 4 days of incubation at 37°C, OD was measured from the bottom of the well in a plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Growth inhibition was calculated from the following formula:

$$\text{Percent OD}_{600} = \frac{\text{Bacteria and Compound (B+C)} - \text{Media Only (M)}}{\text{Bacteria (B)} - \text{Media Only (M)}} \times 100$$

with media only (M) being OD in no growth control wells and bacteria (B) being OD in controls without added antibiotic. The MIC₉₀ was defined as the concentration of antibiotic that caused 90% growth inhibition. Each antibiotic was tested with biological and technical duplicates for every experiment.¹²

Screening for single- and dual-resistant *M. smegmatis* mutants

Single STR- and FLQ-resistant *M. smegmatis* mutants were screened as follows. The wild-type *M. smegmatis* was cultured till the end of log phase, and 200 µL of bacterial culture was plated onto the minimum of 20–30 plates (or more) of 7H9 agar media containing 16 µg/mL of STR for the isolation of STR-resistant colonies and 10 µg/mL of FLQ (fluoroquinolonic acid) for the isolation of FLQ-resistant colonies. The plates were incubated for 3–5 days at 37°C until colonies became visible. Colonies from these plates were picked and sub-cultured in antibiotic-free Middlebrook 7H9 broth, and the bacteria were cultured to the end of log phase for usage. STR-resistant strains with different *rpsL* mutants screened above were used subsequently to obtain STR–FLQ dual-resistant strains by culturing in 7H9 agar media containing 10 µg/mL of FLQ.

Mutation type identification

The resistance-related genes, including *rpsL* for STR and *gyrA* for FLQ, were amplified by polymerase chain reaction (PCR) using DNA extracted from the single- and dual-resistant mutants. The primers used to amplify the whole gene of *rpsL* were 5'-GGT CGG CCC TGG TAT TGTG-3' and 5'-ACG ACG CTG ACG GGA GAAG-3'. Mutations in

the quinolone resistance-determining region (QRDR) of *gyrA* were amplified by primers of 5'-CAT GAG CGT GAT CGT GGG CCG-3' and 5'-CAG AAC CGT GGG CTC CTG CAC-3'. The 50 μ L of PCR mixtures contained 200 μ M of dNTP mixture, 0.2 μ M of each primer, 50 ng of genomic DNA, and 1.25 U of PrimeSTAR HS DNA Polymerase (Takara Biotechnology, Dalian, China). Amplification consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 98°C for 30 s, annealing at 64°C (*rpsL*)/67°C (*gyrA*) for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were purified and sequenced by Thermo Fisher Scientific (Waltham, MA, USA). The results of sequencing were analyzed by BioEdit using the genome sequence of *M. smegmatis* strain mc²155 (NC_018289.1) as the control.

Fitness assay and epistasis measurement

The fitness assay was performed as reported previously.⁷ The mutants were cultured against the wild-type *M. smegmatis* strain mc²155 in antibiotic-free Middlebrook 7H9 media. For each wild-type-mutant pair, between 1 and 4 replicate competition assays were performed. At the start of the experiment, wild-type and mutant cultures were serially diluted to 10⁻⁵–10⁻⁶ and plated on nonselective Middlebrook 7H11 media to estimate the baseline colony-forming units (CFU) count in triplicates. About 100 CFU of bacteria/mL from wild-type and mutant cultures were inoculated in 25 mL of Middlebrook 7H9 media in a 1:1 ratio. The competition cultures were incubated at standard conditions on a shaking incubator at 150 rpm. After 72 h, the same competition cultures were serially diluted to 10⁻⁵–10⁻⁶ and plated on nonselective Middlebrook 7H11 media, as well as diluted to 10⁻⁴–10⁻⁶ and plated on selective Middlebrook 7H11 media to obtain the endpoint CFU counts. The relative fitness of the drug-resistant strain compared with the wild-type and multiplicative model for pairwise epistasis was determined as previously described.⁷ Student's *t*-test was used to detect differences in the mean fitness, and the limit for statistical significance was set at *P*=0.05. Test statistics and estimates were based on 1,000 bootstrap replicates. Statistical analysis was performed with STATA SE/10.

Clinical frequency of STR–FLQ-resistant isolates

Isolated from sputum of pulmonary TB patients in the Public Health Clinical Center of Chengdu, China, from 2010 to 2014, all 213 STR–FLQ dual-resistant *M. tuberculosis* isolates were selected from 2,610 drug-resistant clinical isolates in this study. Genomic DNA was extracted by cetyltrimethyl

ammonium bromide (CTAB) and chloroform/isoamyl alcohol extraction method as previously described.¹³ Primers 5'-GGCCGACAAACAGAACGT-3' and 5'-GTTTCACTCAACTGGGTGAC-3' were used to amplify the full length of STR resistance-related gene *rpsL*; primers 5'-GGC AAC ACC GAG GTC AAA TC-3' and 5'-GCC GTC GTA GTT AGG GAT GAA-3' were used to amplify the QRDR of *gyrA* gene. PCR mixtures were prepared the same as the detection of resistant *M. smegmatis* mutants. PCR program was same as the *gyrA* mutation detection in *M. smegmatis* mutants. PCR products were purified and sequenced by Thermo Fisher Scientific. The results of sequencing were analyzed by BioEdit with the genome sequence of *M. tuberculosis* H37Rv (NC_000962.3) as reference. Additionally, a total of 3,056 whole genome data for clinical *M. tuberculosis* strains were downloaded from The Sequence Read Archive of NCBI (SRA NCBI) database (ERP001072, ERP000192, and SRP018402). The clinical sequence data were aligned with the reference strain H37Rv genome by Bowtie2. The single-nucleotide polymorphisms (SNPs) were identified by Samtools (<https://github.com/samtools/samtools>). Selection of strains with SNPs in *rpsL/gyrA* genes and analysis of mutation types and frequency were performed by a Perl script.

Results

Selection of STR- and FLQ-single- and STR–FLQ-double-resistant mutants

The MIC of the *M. smegmatis* on STR and FLQ was 0.5 and 2 mg/L, respectively (determined by microplate-based growth assay), the screening of resistant *M. smegmatis* mutants was performed by 16 mg/L of STR and 10 mg/L of FLQ. A total of 60 STR- and 89 FLQ-resistant strains were obtained in single-resistant screening, and 90 STR–FLQ dual-resistant mutant strains were obtained by sequential screening on STR-resistant strains. Mutation types of K43R, K43N, K43M, K43T, K88R, and K88E in *rpsL* and D94A, D94Y, A90V, G88A, D94N, and D94G in *gyrA* were identified in single-resistant mutants, respectively. The amino acid sequence alignment of *rpsL* and *gyrA* showed that those mutation sites were highly conserved among eight *Mycobacterium* spp. In addition, positions of mutations K43 and K88 of *rpsL* were marked as STR interaction site (Figure 1), found to be highly conserved between *Escherichia coli* and *M. tuberculosis rpsL* (Figure S1A). Accordingly, we further examined the different combination of mutations in *rpsL* and *gyrA* presenting in dual-resistant mutants (Table 1). Most mutation sites of *gyrA* found in STR–FLQ dual-resistant mutants were consistent with those in FLQ single-resistant mutants.

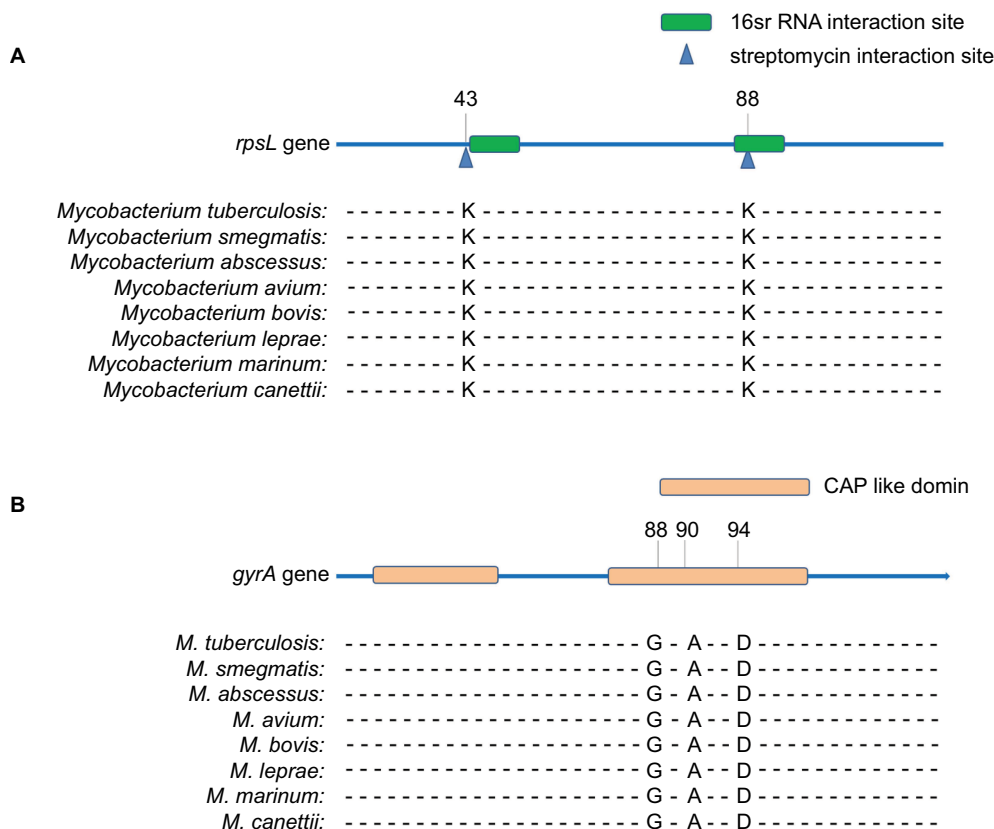


Figure 1 Functional domain and conservation of *rpsL* (A) and *gyrA* (B) mutation sites in *Mycobacterium* spp.

Table 1 Mutation types of single- and dual-resistant *Mycobacterium smegmatis* mutants under the selection of antibiotics

Resistant type	Screening concentration (mg/L)	Number of isolates	Mutation types
STR-R	16	60	<i>rpsL</i> : K43R(28), K43M(7), K43N(4), K43T(2), K88E(14), K88R(4), NM(1)
FLQ-R	10	89	<i>gyrA</i> : D94A(19), D94Y(9), A90V(10), G88A(2), D94N(1), D94G(1), NM(47)
STR-FLQ-R	10	90	K43R: A90V(16), D94G(2), NM(4) K43M: D94G(14), D94A(1), D94Y(1), D94N(1), D89N(1), NM(2) K43N: D94G(9), D94N(1), NM(5) K43T: D94G(1), D94N(1), A90V(2), NM(5) K88R: A90V(2), NM(10) K88E: A90V(2), NM(10)

Abbreviations: FLQ, fluoroquinolones; STR, streptomycin.

Fitness cost of STR-FLQ-resistant mutants

Relative fitness of *M. smegmatis* mutants were obtained by co-cultivation in vitro against their susceptible ancestor. For the STR-resistant mutants, we found that the strains with K43R showed no difference in relative fitness compared with their susceptible ancestor, while K43N, K43M, K43T, and K88E showed a significantly lower relative fitness ($P < 0.05$). Additionally, with the highest relative fitness, K43R mutants presented at the highest frequency (7/15) in STR-screened mutants (Table 1). Interestingly, the strains with K88R mutation showed considerable differences in fitness cost (Figure 2A). For the FLQ-resistant mutants, all mutation types were detected with no difference in relative fitness compared with their susceptible ancestor (Figure 2B). No fitness cost was caused by FLQ-resistant mutations in the resistant strains screened. Except strains harboring K43R and K88R mutations, other STR-FLQ dual-resistant strains showed a significant decrease in relative fitness compared with wild strains (Figure 2C). Significantly negative epistasis was found in the strain with K43M/D94Y, K43M/D94G, and K43T/D94G mutations ($P < 0.05$) (Table S1).

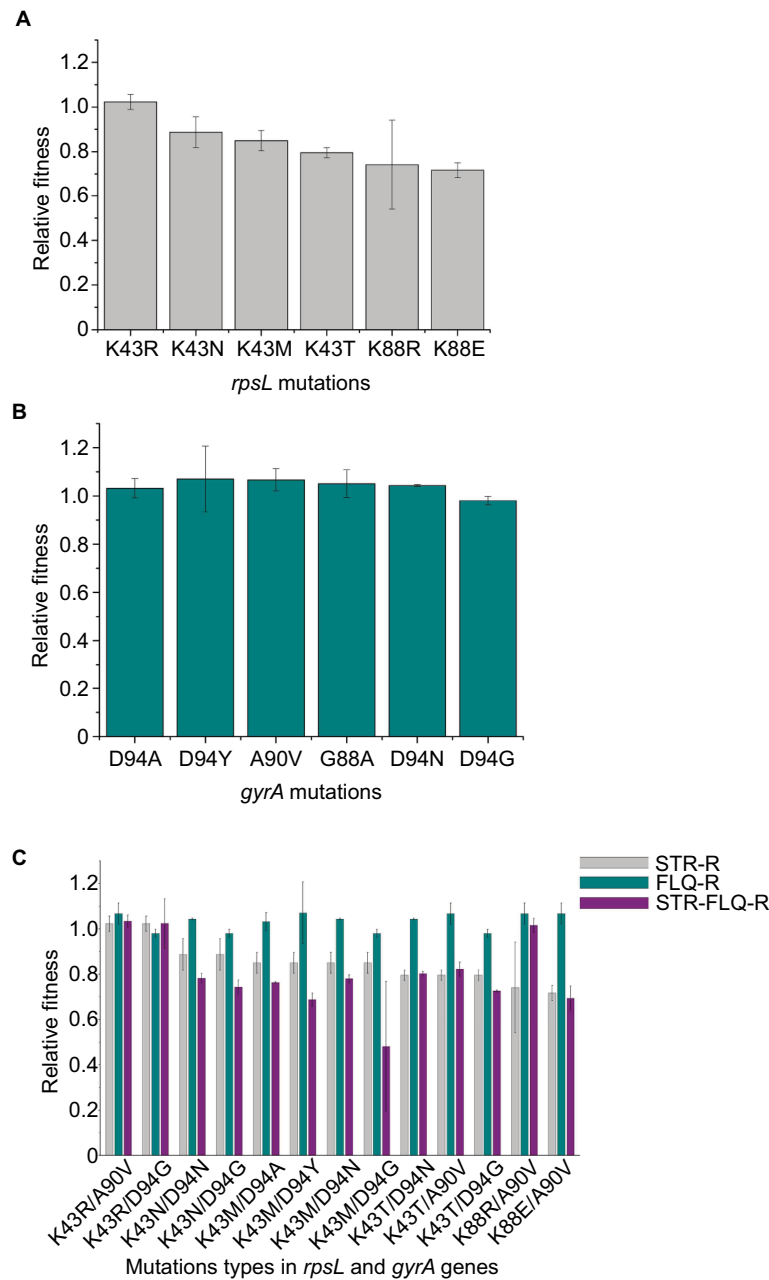


Figure 2 Relative fitness of *Mycobacterium smegmatis* mutants.

Notes: (A) Relative fitness of STR-resistant *M. smegmatis*. (B) Relative fitness of FLQ-resistant *M. smegmatis*. (C) Relative fitness of STR and FLQ dual-resistant *M. smegmatis*. Gray bar is for the STR-resistant *M. smegmatis*, turquoise is for FLQ-resistant, and purple is for the STR–FLQ dual-resistant *M. smegmatis*.

Abbreviations: FLQ, fluoroquinolones; STR, streptomycin.

Remarkably, the model of mutations appearing in *rpsL* and *gyrA* in this study (Figure S1) allows to have an insight on the structural evidence of dispensable feature for these mutations. There are 69% identities between *M. tuberculosis* and *E. coli rpsL* (Figure S2). Mutation sites of *M. tuberculosis rpsL* could be seen through the structure of *E. coli rpsL* (Protein Data Bank accession number 5U4I). Indeed, K43 and K88 of *M. tuberculosis rpsL* are located closely in the conformation of *E. coli* 30S ribosomal protein S12 (5U4I as the structure of *E. coli* entire ribosomal complex).¹⁴ Similarly,

the G88, A90, and D94 of *M. tuberculosis gyrA*, crucial for its proper function, sit at the interacting area of chain A and chain B of 3IFZ, which is the crystal structure of *M. tuberculosis* DNA gyrase.¹⁵

Clinical epistasis in STR–FLQ-resistant *M. tuberculosis*

Among the 213 STR–FLQ dual-resistant clinical isolates collected, we identified 122 isolates with mutations in both *rpsL* and *gyrA* genes and 25 isolates with no mutations

in them. The other 17 and 49 isolates were detected with mutations in *rpsL* and *gyrA*, respectively. Four mutation types, such as K43R (n=107), K88R (n=30), K88T (n=1), and K88M (n=1), were seen in *rpsL*, while eight mutation types, such as D94G (n=72), A90V (n=41), D94A (n=17), S91P (n=12), D94Y (n=15), D94N (n=12), D94H (n=2), and G88C (n=1), were seen in *gyrA* (Table 2). Several mutation types in *rpsL* (K43N, K43M, K43T, and K88E), present in STR-resistant *M. smegmatis* mutants, were not identified in clinical *M. tuberculosis* isolates. Similarly, the *gyrA* mutation present in FLQ-resistant *M. smegmatis* strains (G88A) was not identified in *M. tuberculosis* strains. Among the *M. tuberculosis* strains harboring *rpsL* and *gyrA* dual mutations, the most frequent combinatorial mutation types were K43R/D94G (n=37) and K43R/A90V (n=24) (Figure 3A). By searching 3,056 *M. tuberculosis* genome sequence in global SRA NCBI database, we found 148 strains with *rpsL* and *gyrA* combinatorial mutations (Figure 3B). Similar to our local clinical data, only K43R and K88R mutation types were found in *rpsL* in *M. tuberculosis*, with D94G, A90V,

D94A, S91P, D94Y, and D94N being the predominant ones (79.7%) in *gyrA*. Based on the information available, K43R and D94G mutations exhibited the highest frequency in *rpsL* and *gyrA*, respectively.

Discussion

The development of drug resistance in *M. tuberculosis* is commonly influenced by different factors, such as the lineages preferentially associated with resistance to multiple drugs,¹⁶ enhanced heterogeneity at low pH,¹⁷ and sub-lethal antibiotic treatment.¹⁸ Epistatic interactions in strains of different genetic background, drug-resistant mutations, and compensatory mutations could play a role in the emergence of drug-resistant *M. tuberculosis*.¹⁹ In our study, significant epistasis was found in mutations occurring in *rpsL* and *gyrA* of mycobacteria.

The genes of *rpsL* and *gyrA* have been widely reported to be related to STR and FLQ resistance in *M. tuberculosis*, respectively.²⁰ Mutations in the DNA gyrase genes (*gyrA* and *gyrB*) may lead to the resistance to FLQ. However, most of the clinical isolates showed mutations mainly on codons 90, 91, and 94, which are located in the QRDR of *gyrA*. Thus, the FLQ-resistant mutants were analyzed by sequencing the QRDR of *gyrA* in the present study. In our study, 59/60 STR-resistant strains selected from susceptible *M. smegmatis* were identified to harbor the mutations in codon 43 and 88 of *rpsL*, suggesting that the nucleotide change in *rpsL* was the main cause of STR resistance in spontaneous mutation in vitro, which was similar for STR-resistant *M. smegmatis* strains screened in the previous study.²¹ However, only 42/89 FLQ-resistant *M. smegmatis* strains were detected with mutations in *gyrA* and this implied that other important mechanisms might exist in the development of resistance to FLQ, for example, the phenotypic resistance to FLQ by mycobacterial mistranslation.²² In previous report by Borrell et al,⁷ only four mutation types were found in *gyrA*, whereas more *gyrA* mutations were detected in our screening, providing a more comprehensive information on their functions. Various combinations of *rpsL* and *gyrA* mutations were detected in dual-resistant *M. smegmatis* screening, suggesting that some kinds of preferences might exist favoring the development of dual-mutations.

It has been generally accepted that the acquisition of drug resistance typically confers a reduction in fitness when the antibiotic is absent.²³ However, drug-resistant-related mutations with low or no fitness cost were reported recently in *M. tuberculosis* and *Salmonella typhi*,^{24–26} and the evolution of compensatory mutations may further contribute to

Table 2 Gene mutations in clinical STR- and FLQ-resistant *Mycobacterium tuberculosis* isolates

Mutation type	<i>rpsL</i>	<i>gyrA</i>	Number of isolates	
<i>rpsL</i> (K43R)/ <i>gyrA</i>	aag@agg K43R	gac@ggc D94G	37	
	aag@agg K43R	gcg@gtg A90V	24	
	aag@agg K43R	gac@gcc D94A	8	
	aag@agg K43R	tcg@ccg S91P	9	
	aag@agg K43R	gac@tac D94Y	10	
	aag@agg K43R	gac@aac D94N	4	
	aag@agg K43R	gac@aac D94H	2	
	<i>rpsL</i> (K88R)/ <i>gyrA</i>	aag@agg K88R	gac@ggc D94G	11
aag@agg K88R		gcg@gtg A90V	7	
aag@agg K88R		gac@gcc D94A	5	
aag@agg K88R		tcg@ccg S91P	1	
aag@agg K88R		gac@tac D94Y	1	
aag@agg K88R		gac@aac D94N	2	
<i>rpsL</i> (K88T)/ <i>gyrA</i> Single mutation		aag@acg K88T	gac@ggc D94G	1
		aag@agg K43R	NM	13
	aag@agg K88R	NM	3	
	aag@atg K88M	NM	1	
	NM	gac@ggc D94G	23	
	NM	gcg@gtg A90V	10	
	NM	gac@gcc D94A	3	
	NM	tcg@ccg S91P	2	
	NM	gac@tac D94Y	4	
	NM	gac@aac D94N	6	
	NM	ggc@tgc G88C	1	
	No mutation	NM	25	

Note: NM, no mutation detected.

Conclusion

The fitness of dual-resistant strains was primarily determined by the mutation type of *rpsL*, which was verified by clinical frequency distortion of drug-resistant mutations. Although only one negatively epistatic mutation combination of K43M/D94Y was detected in our study and significant absence of this combination was found in clinical investigation, the further work should be performed to explore the negatively epistatic mutation combination in other anti-TB drugs. Understanding how the resistance conferring mutations interact with negative epistasis could help us optimize the treatment of combined use of drugs and thus reduce the possibility of drug resistance emergence.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. WHO. *Global Tuberculosis Report. 2016*. Geneva: World Health Organization; 2016.
2. Dheda K, Gumbo T, Gandhi NR, et al. Global control of tuberculosis: from extensively drug-resistant to untreatable tuberculosis. *Lancet Respir Med*. 2014;2(4):321–338.
3. Lehner B. Molecular mechanisms of epistasis within and between genes. *Trends Genet*. 2011;27(8):323–331.
4. Breen MS, Kemena C, Vlasov PK, Notredame C, Kondrashov FA. Epistasis as the primary factor in molecular evolution. *Nature*. 2012;490(7421):535–538.
5. Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I. Positive epistasis drives the acquisition of multidrug resistance. *PLoS Genet*. 2009;5(7):e1000578.
6. Müller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Trends Genet*. 2013;29(3):160–169.
7. Borrell S, Teo Y, Giardina F, et al. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol Med Public Health*. 2013;2013(1):65–74.
8. Committee TT. Streptomycin treatment of pulmonary tuberculosis: a medical research council investigation. *Br Med J*. 1948;2(4582):769–782.
9. Pourakbari B, Mamishi S, Mohammadzadeh M, Mahmoudi S. First-line anti-tubercular drug resistance of *Mycobacterium tuberculosis* in Iran: a systematic review. *Front Microbiol*. 2016;7:1139.
10. Caminero JA, Sotgiu G, Zumla A, Migliori GB. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *Lancet Infect Dis*. 2010;10(9):621–629.
11. Mir M, Prusic S, Kang CM, et al. Mycobacterial gene *cuvaA* is required for optimal nutrient utilization and virulence. *Infect Immun*. 2014;82(10):4104–4117.
12. Singh AK, Carette X, Potluri LP, et al. Investigating essential gene function in *Mycobacterium tuberculosis* using an efficient CRISPR interference system. *Nucleic Acids Res*. 2016;44(18):e143.
13. Guo JH, Xiang WL, Zhao QR, et al. Molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Sichuan Province in China. *Jpn J Infect Dis*. 2008;61(4):264–268.
14. Zeng F, Chen Y, Remis J, et al. Structural basis of co-translational quality control by ArfA and RF2 bound to ribosome. *Nature*. 2017;541(7638):554–557.
15. Piton J, Petrella S, Delarue M, et al. Structural insights into the quinolone resistance mechanism of *Mycobacterium tuberculosis* DNA gyrase. *PLoS One*. 2010;5(8):e12245.
16. Ford CB, Shah RR, Maeda MK, et al. *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet*. 2013;45(7):784–790.
17. Jenkins C, Bacon J, Allnut J, et al. Enhanced heterogeneity of *rpoB* in *Mycobacterium tuberculosis* found at low pH. *J Antimicrob Chemother*. 2009;63(6):1118–1120.
18. Kohanski MA, DePristo MA, Collins JJ. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell*. 2010;37(3):311–320.
19. Borrell S, Gagneux S. Strain diversity, epistasis and the evolution of drug resistance in *Mycobacterium tuberculosis*. *Clin Microbiol Infect*. 2011;17(6):815–820.
20. Walker TM, Kohl TA, Omar SV, et al. Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: a retrospective cohort study. *Lancet Infect Dis*. 2015;15(10):1193–1202.
21. Pelchovich G, Zhuravlev A, Gophna U. Effect of ribosome-targeting antibiotics on streptomycin-resistant *Mycobacterium* mutants in the *rpsL* gene. *Int J Antimicrob Agents*. 2013;42(2):129–132.
22. Javid B, Sorrentino F, Toosky M, et al. Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. *Proc Natl Acad Sci U S A*. 2014;111(3):1132–1137.
23. Andersson DI. Persistence of antibiotic resistant bacteria. *Curr Opin Microbiol*. 2003;6(5):452–456.
24. Lee JH, Ammerman NC, Nolan S, et al. Isoniazid resistance without a loss of fitness in *Mycobacterium tuberculosis*. *Nat Commun*. 2012;3(2):753.
25. Spies FS, von Groll A, Ribeiro AW, et al. Biological cost in *Mycobacterium tuberculosis* with mutations in the *rpsL*, *rrs*, *rpoB*, and *katG* genes. *Tuberculosis*. 2013;93(2):150–154.
26. Baker S, Duy PT, Nga TV, et al. Fitness benefits in fluoroquinolone-resistant *Salmonella* Typhi in the absence of antimicrobial pressure. *Elife*. 2013;2(49):e01229.
27. Shcherbakov D, Akbergenov R, Matt T, Sander P, Andersson DI, Böttger EC. Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the in vivo evolution of aminoglycoside resistance in *Mycobacterium tuberculosis*. *Mol Microbiol*. 2010;77(4):830–840.
28. Vogwill T, Kojadinovic M, MacLean RC. Epistasis between antibiotic resistance mutations and genetic background shape the fitness effect of resistance across species of *Pseudomonas*. *Proc Biol Sci*. 2016;283(1830):20160151.
29. Durão P, Trindade S, Sousa A, Gordo I. Multiple resistance at no cost: rifampicin and streptomycin a dangerous liaison in the spread of antibiotic resistance. *Mol Biol Evol*. 2015;32(10):2675–2680.
30. Stower H. Molecular evolution: epistasis prevails. *Nat Rev Genet*. 2012;13(12):828.
31. Salvatore PP, Becerra MC, Abel zur Wiesch P, et al. Fitness costs of drug resistance mutations in multidrug-resistant *Mycobacterium tuberculosis*: a household-based case-control study. *J Infect Dis*. 2016;213(1):149–155.

Supplementary materials

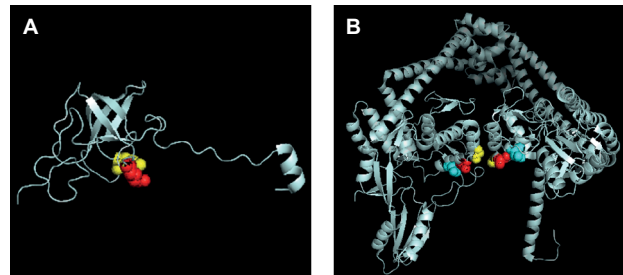


Figure S1 Model of mutations appearing in *rpsL* and *gyrA* in this study.

Notes: (A) Structure of *Escherichia coli rpsL* (Protein Data Bank accession number 5U4I). The position of K43 residue (yellow) and K88 residue (red) screened in this study was highly conserved between *E. coli* and *Mycobacterium tuberculosis*. (B) *M. tuberculosis gyrA* (Protein Data Bank accession number 3IFZ). The position of G88 residue (yellow), A90 residue (red), and D94 residue (cyan) screened in present study is shown. Amino acids were placed in the cartoon structure in dots format using the PyMOL Viewer.

Range 1: 21–144

	Score	Expect method	Identities	Positives	Gaps	Frame
	180 bits(457)	8e-65()	Compositional matrix adjust.	86/124(69%)	102/124(82%)	0/124(0%)
<i>Mycobacterium tuberculosis rpsL</i> 1			MPTIQQLVVRKGRDKISKVKTAAALKGSPQRRGVCTRVYTTTPKKPNSALRKVARVKLTSQ	*		60
<i>Escherichia coli rpsL</i> 21			M T+ QLVRK R K++K AL+ PQ+RGVCTRVYTTTPKKPNSALRKV RV+LT+ MATVNQLVVRKPRARKVAKSNVPALEACPQKRGVCTRVYTTTPKKPNSALRKVCRVRLTNG			80
<i>M. tuberculosis rpsL</i> 61			VEVTAYIPGEGHNLQEHSMVLVRGGRVKDLPGVRYKIIRGSLDTQGVKNRKQARSRYGAK			120
<i>E. coli rpsL</i> 81			EVT+YI GEGHNLQEHSMVLVRGGRVKDLPGVRY +RG+LD GVK+RKQARS+YG K FEVTSYIGGEGHNLQEHSMVLIRGGRVKDLPGVRYHTVRGALDCSGVKDRKQARSKYGVK	K43		140
<i>M. tuberculosis rpsL</i> 121	KEKG	124		*		
	+ K			K88		
<i>E. coli rpsL</i> 141	RPKA	144				

Figure S2 The alignment of *rpsL* in *M. tuberculosis* and *E. coli*.

Table S1 Epistasis (ϵ) in mutants resistant to STR and FLQ

Sample	STR–FLQ resistance		ϵ	SD of ϵ
	<i>rpsL</i> SNP	<i>gyrA</i> SNP		
M _s _SFI6R12	K43R	A90V	−0.057	0.021
M _s _SFI6R14	K43R	D94G	0.021	0.020
M _s _SFI6R210	K43N	D94N	−0.143	0.080
M _s _SFI6R22	K43N	D94G	−0.127	0.076
M _s _SFI6R262	K43M	D94A	−0.114	0.052
M _s _SFI6R265	K43M	D94Y	−0.222	0.054
M _s _SFI6R260	K43M	D94N	−0.106	0.052
M _s _SFI6R261	K43M	D94G	−0.352	0.049
M _s _SFI6R18	K43T	D94N	−0.027	0.015
M _s _SFI6R19	K43T	A90V	−0.027	0.015
M _s _SFI6R13	K43T	D94G	−0.055	0.014
M _s _SFI6R204	K88R	A90V	0.224	0.283
M _s _SFI6R217	K88E	A90V	−0.072	0.038

Abbreviations: FLQ, fluoroquinolones; STR, streptomycin.

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