Fitness Cost and Compensatory Evolution in Levofloxacin-Resistant *Mycobacterium aurum*

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ABSTRACT We isolated spontaneous levofloxacin-resistant strains of *Mycobacterium aurum* to study the fitness cost and compensatory evolution of fluoroquinolone resistance in mycobacteria. Five of six mutant strains with substantial growth defects showed restored fitness after being serially passaged for 18 growth cycles, along with increased cellular ATP level. Whole-genome sequencing identified putative compensatory mutations in the *glgC* gene that restored the fitness of the resistant strains, presumably by altering the bacterial energy metabolism.

KEYWORDS levofloxacin, resistance, fitness cost, compensatory mutation, mycobacteria

Third-generation fluoroquinolones (FQs) levofloxacin and moxifloxacin play a critical role in the treatment of multidrug-resistant tuberculosis (1), and the development of fluoroquinolone resistance (FQ-R) in *Mycobacterium tuberculosis* dramatically reduces the rates of treatment success (2). FQs inhibit the action of DNA gyrase, which is composed of two A and two B subunits, encoded by *gyrA* and *gyrB*, respectively (3). Mutations in two short regions of *gyrA* and *gyrB*, known as the “quinolone-resistance-determining regions” (QRDRs) have been shown to cause FQ-R. In *M. tuberculosis*, the most common FQ-R mutations alter codons 90 and 94 of *gyrA*, but substitutions are also found in codons 74, 88, 89, and 91 (4, 5). Drug resistance mutations generally impart a fitness cost manifested as reduced growth rates and lower virulence, which could be ameliorated by second-site compensatory mutations (6–8). Such compensatory evolution could play a pivotal role in the maintenance and spread of drug-resistant bacteria (9). Previous study has demonstrated low clustering rates for FQ-resistant clinical *M. tuberculosis* strains (10), suggesting that FQ-R mutations may somehow make them less likely to be transmitted, but little is known about the fitness costs of FQ-R and the evolution of possible compensatory mutations. Here, we used *Mycobacterium aurum* as a model organism for studying the fitness cost and compensatory evolution of FQ-R in *M. tuberculosis*. Compared to other mycobacteria used as models for tuberculosis research, *M. aurum* does not aggregate, which facilitates precise colony enumeration (11). Although fast growing and nonpathogenic, *M. aurum* is like *M. tuberculosis* for FQ-R, and its QRDRs of *gyrA* and *gyrB* show amino acid identities of 95 and 97.56%, respectively, with those of *M. tuberculosis*.

We generated a total of 144 levofloxacin-resistant *M. aurum* strains from three rounds of selection with 2-fold increasing concentrations of levofloxacin. As shown in Table 1, 45 single mutants were isolated from the wild-type (WT) strain, while the 92 double and 7 triple mutants were derived from strains with single and double mutations, respectively. Primers for amplifying and sequencing of the QRDRs of *gyrA* and

Citation Pi R, Liu Q, Takiff HE, Gao Q. 2020. Fitness cost and compensatory evolution in levofloxacin-resistant *Mycobacterium aurum*. Antimicrob Agents Chemother 64:e00224-20. <https://doi.org/10.1128/AAC.00224-20>.

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Received 5 February 2020

Returned for modification 5 March 2020

Accepted 25 May 2020

Accepted manuscript posted online 1 June 2020

Published 22 July 2020

TABLE 1 Mutation and levofloxacin MICs of levofloxacin-resistant *M. aurum* strains

No. of mutations	Prior mutation ^a	Secondary mutation ^a		Selective concn (μg/ml) ^b	MIC (μg/ml)	No. of strains ^c
		<i>gyrA</i>	<i>gyrB</i>			
Single mutation	None	G88C	WT	0.2	0.4	3*#
		G88D	WT	0.2–0.4	6.4	9*
		G89D	WT	0.4	3.2	2*
		S90L	WT	0.05–0.4	0.8	10*
		D94N	WT	0.05–0.4	0.8	11*#
		D94G	WT	0.2	0.8	2*#
		D94Y	WT	0.2	0.4	4*#
		WT	R485L	0.2	0.4	4*
Double mutations	<i>gyrA</i> G88C	D94Y	WT	1.6	3.2	1
		D94Y	WT	0.8–6.4	25.6	2*
		D94A	WT	1.6–3.2	12.8	3*
		D94G	WT	0.8–3.2	25.6	7*
		D94N	WT	0.8–6.4	25.6	8
	<i>gyrA</i> S90L	WT	D500N	1.6	6.4	1*
		G88C	WT	0.8	1.6	1
		G88D	WT	0.4–3.2	6.4	2*
		G89N	WT	0.8	3.2	1
		D89G	WT	0.8	1.6	1*
	<i>gyrA</i> D94N	S90L	WT	0.8–3.2	25.6	17
		S90A	WT	0.8	25.6	3
		A74S	WT	0.8	1.6	2
		G88D	WT	0.8	3.2	1
		D89N	WT	0.8	3.2	2*
	<i>gyrA</i> D94G	S90L	WT	0.8	12.8	7*
		S90A	WT	0.8	12.8	1*
		S91P	WT	0.8	3.2	1#
		WT	D500N	0.8	3.2	1*
		WT	L538F	0.8	3.2	2*
	<i>gyrA</i> D94Y	G88C	WT	0.8–1.6	3.2	2*
		D89N	WT	0.8	3.2	3*
		D89G	WT	0.8	3.2	1
		S90L	WT	0.8	25.6	3
		S91P	WT	0.8	3.2	1
	<i>gyrB</i> R485L	WT	R485H	0.8	3.2	2*
		G88D	WT	0.8–1.6	3.2	13
		S90L	WT	0.8	3.2	1*
		D94G	WT	0.8	1.6	1*
		D94Y	WT	0.8	3.2	1*
Triple mutations	<i>gyrA</i> S90L + <i>gyrA</i> D94A	WT	L538F	12.8	51.2	1*
		WT	D500H	25.6	51.2	2*
	<i>gyrA</i> S90L + <i>gyrB</i> D500N	WT	D94N	25.6	51.2	1*
		WT	D94Y	12.8–25.6	51.2	3*

^aAll *gyrA* and *gyrB* mutations were transformed to corresponding codon substitutions in *M. tuberculosis*.^bThe concentrations used to select levofloxacin-resistant *M. aurum* mutants increased twofold.^cAsterisks (*) indicate strains subjected to growth rate measurements in a preliminary screen for levofloxacin-resistant strains with decreased fitness, and pound signs (#) indicate the mutations in the indicated strains were observed in clinical *M. tuberculosis* strains.

gyrB are described in Table S1 in the supplemental material. Levofloxacin MIC values were determined using broth microdilution method (12). As shown in Table 1, most levofloxacin resistance mutations occurred in codons 90 and 94 of *gyrA*, and resistance levels increased with mutation accumulation. To further investigate whether FQ-R influences the bacterial fitness, we analyzed the growth kinetics by recording the optical density at 600 nm (OD₆₀₀) of a WT control and levofloxacin-resistant *M. aurum* strains with different mutations. Most of the mutant strains (23/29, 79%) had little or no growth defect (Fig. 1a), but six mutant strains showed growth rates substantially less than that of the WT strain (Fig. 1b). The *gyrA* mutations in these strains were G88C, G88D, D94N+D89G, D94G+D89N, D94Y+D89N, and D94Y+G88C (Table 1). To quantify the relative fitness of these six mutant strains compared to WT strain, we performed an *in vitro* competition assay, as previously described (13), by inoculating equal

FIG 1 Fitness costs and compensation of mutant *M. aurum* strains. (a and b) Graphs show the growth curves of *M. aurum* strains in nonantibiotic 7H9 broth. The OD₆₀₀ is plotted as a function of time. Mutations at codons 88, 89, 90, 91, and 94 are located in the *gyrA* gene, and mutations at codons 500 and 538 are located in the *gyrB* gene. The WT and single mutants are indicated by a circle, while double and triple mutants are indicated by triangles and asterisks, respectively. (c and d) Growth curves of *M. aurum* strains after 0 (solid lines) and 18 (dashed lines) growth cycles in nonantibiotic and 0.2 μ g/ml of levofloxacin 7H9 broth, respectively. (e and f) Relative fitness (e) and ATP levels (f) of *M. aurum* strains after 0 (dark shading) and 18 (light shading) growth cycles, respectively. The results show the means $\pm$ the standard deviations of three independent experiments, and the exact *P* values are listed above the pairs of data (paired *t* test).

volumes of WT and mutant cultures (OD₆₀₀ = 1) at a 1:100 dilution, and counting colonies at baseline (0 h) and endpoint (72 h). All six slow-growing mutants showed significantly lower fitness compared to the WT strain (Fig. 1e), but the strain that was least fit had the *gyrA* G88C substitution, as also seen in *Mycobacterium smegmatis* (14). Interestingly, the fitness of this G88C mutant was partially restored by the acquisition of a second *gyrA* D94Y substitution, suggesting an intragenic compensatory mechanism.

We then experimentally evolved the six slow-growing mutants in the laboratory, with the WT strain as a control. Eight independent cultures for each strain were grown in antibiotic-free 7H9 broth for 3 days and then serially passaged by transferring 3 μ l of culture into 3 ml of fresh medium to initiate the next 3-day growth cycle. After 18 growth cycles, five of the six evolved mutant strains showed growth rates that were restored to that of the WT and improved fitness compared to their mutant parents (Fig. 1c to e). The only mutant that did not evolve restored fitness after 18 growth cycles was the strain with the single G88C substitution. Illumina whole-genome sequencing (WGS) found that all five evolved fitness-restored strains convergently had frameshift insertions in the *glgC* gene, which encodes an ADP-glucose pyrophosphorylase (AGPase) (Table S2). The evolved strains maintained the same MICs as their slower growing

ancestors, indicating that the compensatory mutations had no impact on drug resistance (Table S2). In addition, WGS identified mutations in 12 other genes in the evolved strains, including some from the passaged WT strain (Table S3), but none of these mutations were found in evolved strains from more than one original mutant.

FQ-R mutations have been reported to alter the enzymatic activity of the ATP-dependent DNA gyrase (15). Because the AGPase, encoded by *glgC* gene, is involved in the ATP-consuming process of glycogen synthesis (16), frameshift insertions that inactivate the *glgC* gene might reduce glycogen synthesis and therefore ATP consumption. Consistent with this explanation, cellular ATP concentrations, measured using an ATP assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions, were significantly higher in all faster growing evolved strains than in their pre-evolved mutant parents (paired *t* test, $P < 0.01$) (Fig. 1f). The higher ATP levels could perhaps increase the activity of the ATP-dependent DNA gyrase, thus compensating for a putatively reduced functional gyrase capacity in the low fitness mutants. Since the GlgC proteins of *M. aurum* and *M. tuberculosis* are highly similar (identity, 91.34%), we searched for *glgC* mutations in a data set of more than 700 clinical FQ-resistant *M. tuberculosis* strains but identified only one strain, with *gyrA* D94G plus *gyrB* A504V substitutions, that had an insertion mutation in the *glgC* gene. It is possible that the putative compensatory mutations in the *glgC* gene are not found in clinical *M. tuberculosis* isolates because low fitness FQ-R mutant strains would not survive long enough in a clinical setting to develop a compensatory mutation. However, occasional clinical strains are found with the fitness-costing mutations we describe, but they may have compensatory mutations that, due to differences in the biology of *M. tuberculosis*, are distinct from the AGPase inactivating insertions found in *M. aurum*. The reports that FQ-R strains are less frequently transmitted could indicate that the more common FQ-R mutations have subtle fitness costs that we could not appreciate through *in vitro* studies of *M. aurum* but may affect the relative abilities of *M. tuberculosis* strains to cause disease.

In conclusion, we demonstrated that some levofloxacin-resistant *M. aurum* strains with mutations in *gyrA* showed fitness costs that were compensated with the acquisition of insertions into the *glgC* gene. The fitness compensated strains showed higher ATP concentrations than the WT strain, perhaps due to decreased ATP consumption resulting from the absence of the *glgC* encoded AGPase. This study represents a first step toward a better understanding of the fitness effects of FQ-R mutations in mycobacteria. Further studies are warranted to investigate the molecular basis of fitness costs and the compensatory mechanisms in FQ-R mycobacteria.

Data availability. The WGS data have been submitted to the Sequence Read Archive of the National Center for Biotechnology Information as recalibrated BAM files (accession number [PRJNA598519](https://www.ncbi.nlm.nih.gov/sra/PRJNA598519)).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This study was supported by the Natural Science Foundation of China (81661128043 and 81871625 to Q.G. and 81701975 to Q.L.) and the National Science and Technology Major Project of China (2017ZX10201302 and 2018ZX10715012).

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