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Drug resistance, fitness and compensatory mutations in Mycobacterium tuberculosis

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ABSTRACT

For tuberculosis to be eradicated, the transmission of Multi-Drug-Resistant and eXtensively Drug Resistant strains of *Mycobacterium tuberculosis* (MDR and XDR-TB) must be considerably reduced. Drug resistant strains were initially thought to have reduced fitness, and the majority of resistant strains may actually have compromised fitness because they are found in only one or a few patients. In contrast, some MDR/XDR-TB strains are highly transmitted and cause large outbreaks. Most antibiotics target essential bacterial functions and the mutations that confer resistance to anti-TB drugs can incur fitness costs manifested as slower growth and reduced viability. The fitness costs vary with different resistance mutations and the bacilli can also accumulate secondary mutations that compensate for the compromised functions and partially or fully restore lost fitness. The compensatory mutations (CM) are different for each antibiotic, as they mitigate the deleterious effects of the specific functions compromised by the resistance mutations. CM are generally more common in strains with resistance mutations incurring the greatest fitness costs, but for RIF resistance, CM are most frequent in strains with the mutation carrying the least fitness cost, Ser450Leu. Here, we review what is known about fitness costs, CM and mechanisms of resistance to the drugs that define a strain as MDR or XDR-TB. The relative fitness costs of the resistance mutations and the mitigating effects of CM largely explain why certain mutations are frequently found in highly transmitted clusters while others are less frequently, rarely or never found in clinical isolates. The CM illustrate how drug resistance affects bacteria and how bacteria evolve to overcome the effects of the antibiotics, and thus a paradigm for how mycobacteria can evolve in response to stress.

1. Introduction

According to World Health Organization (WHO) [1,2], among the approximately 10 million people who develop tuberculosis each year, about half a million have rifampicin resistant (RIF-R) strains of *Mycobacterium tuberculosis* (*M. tb)*, 78% of which are also resistant to isoniazid (INH) and termed Multi-Drug-Resistant, or MDR-TB. Of these, about 5–8% have additional resistance to a fluoroquinolone (FQ) and one of the injectable drugs (capreomycin, kanamycin or amikacin), and were termed extensively drug resistant, or XDR-TB. MDR-TB strains only resistant to a FQ are termed pre-XDR-TB. Recently the WHO changed the definition of XDR-TB to indicate resistance to the FQs levofloxacin or moxifloxacin and at least one of the other group I agents – bedaquiline or linezolid [3]. However, as the studies reviewed here were performed before the definition was altered, this review will use the previous definition of XDR-TB. MDR and XDR-TB strains require longer, more expensive treatments than Drug Sensitive (DS) strains, and only about 57% [2,4] of MDR-TB patients who receive treatment are cured, although recently introduced regimens have shown cure rates up to 80% [5–8]. With good reason, drug resistant (DR) strains are seen as a serious impediment to TB control and eradication.

Shortly after Isoniazid (INH) began to be used against tuberculosis (TB) in the 1950s, Middlebrook found that most INH resistant *M. tb* strains were less virulent in Guinea pigs [9–12], and based largely on

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these observations, it was believed that DR strains were inherently weaker, or less fit, than DS strains. Because antibiotics generally inhibit enzymes essential for bacterial survival, it is logical that resistance-conferring mutations altering these critical enzymes could result in a loss of fitness: the bacteria grow slower, are transmitted less often and perhaps cause less severe disease [13–17]. However, resistant strains can also acquire secondary, compensatory mutations (CM) that partially or fully restore the fitness cost of the drug resistance conferring mutations. The CM vary depending upon the antibiotic because they mitigate the specific functions compromised by the resistance mutations [18]. Although compensatory mutations are generally more common in strains with resistance mutations incurring the greatest fitness costs, the opposite is true with RIF-R mutations, as described below.

Many MDR and XDR strains are seen in only one or a few patients and may, in fact, be less fit than most DS strains [19], but in regions with high rates of DR-TB, a large part of the MDR burden is often caused by a few dominant, highly transmitted clades of closely related strains [20]. Globally, it is currently estimated that transmission of MDR-TB strains is responsible for over 70% of MDR-TB cases [21]. Is it possible that the differences in strain transmissibility are related to the balance between the fitness costs of the particular mutations conferring resistance and the ability of acquired compensatory mutations to restore the fitness of the resistant strains? To address this question, we review here what is known about the fitness costs of the DR mutations that make a strain MDR or XDR-TB, and the compensatory mutations that fully or partially restore the fitness lost with these DR mutations (Table 1). The studies in this review were largely *in vitro*, but the putative effects of the DR and CM mutations on the transmission of MDR/XDR-TB outbreak strains are examined in a companion review [22].

2. Isoniazid

Isoniazid (INH) has been a staple of first-line anti-tuberculosis therapy since shortly after it was discovered (actually rediscovered [23]) in the 1950's. After Middlebrook found that most INH resistant strains appeared to be less virulent in Guinea pigs [9–11,24], some TB labs tested for INH resistance by injecting isolates into Guinea pigs, and if the animals survived, the strain was presumed to be INH resistant (INH-R) (personal communication, Instituto Malbran, Buenos Aires, Argentina). Early studies noted that some INH-R *M. tb* strains [15] also lacked catalase-peroxidase activity [10], and forty years later Zhang et al. explained this relationship by introducing an intact copy of the *katG* gene into a peroxidase deficient INH-R strain and restoring both full virulence and INH sensitivity [25–27]. INH is given as a prodrug that must be oxidized by the bacterial catalase/peroxidase KatG to an active form that interacts with NAD+ [25,26,28–30]. The INH/NAD + complex inserts into the NADH binding pocket of InhA, the target protein of INH, thereby inhibiting its activity. InhA is an enoyl acyl carrier protein (ACP) reductase involved in the synthesis of mycolic acids. It uses NADH as a cofactor [31,32], but the affinity of InhA for the INH/NAD + complex is 200-fold greater than for NADH. Any mutation that reduces the ability of KatG to activate INH will confer INH resistance [33], and more than half of all INH-R isolates of *M. tb* have mutations in *katG* [34], which generally confer high-level INH resistance. A variety of amino acid substitutions have been described that reduce the activity or stability of the KatG enzyme [35], and occasionally the *katG* gene is partially or completely deleted [28]. A recent study found that the KatG proteins of other mycobacteria are less effective at activating INH, which helps explains why INH is only effective against *M. tb* [36].

There are two other types of INH-R mutations involving InhA that confer lower-level resistance. Mutations that increase the activity of the

Table 1
Resistance and compensatory mutations for the antibiotic resistance defining MDR/XDR-TB.

Antibiotic	Gene	Resistance or Compensatory	Site of Mutation	Effect on Fitness	In highly transmitted strains?
INH	*katG*	Resistance	Ser315Thr	minor	often, esp. Modern Beijing
			other AA's & deletions	large	
	inhA promoter	Resistance	−8, −15, −17, or −47	unclear	yes
	inhA structure	Resistance	AA 94 or 194	unclear	yes
	ahpC promoter	Compensatory ?	Promoter up	unclear	not exclusively
RIF	*rpoB RRDR*	Resistance	Ser450Leu	minor	most common; high freq CM
			Asp435Gly	severe	
			AA 445 & others	moderate	
	rpoC	Compensatory	Phe452Leu, Val483Gly & other sites	restores fitness	Yes
	rpoA	Compensatory	Several sites	restores fitness	Yes
	rpoB	Compensatory	Ile1106Thr & others	presumed to restore	Yes
STR	*rpsL*	Resistance	Lys43Arg	minimal	Yes
			Lys43Thr	transl. hyperaccuracy	No
			Lys43Asp	transl. hyperaccuracy	No
			Lys88Arg	minimal	Yes
			Lys88Glu	moderate	No
		Compensatory	*rpsD* & rpsE	partially restores fitness by reducing translation Accuracy	No
	16S rRNA (*rrs*)	Resistance	nt 512, 513, or 516	minimal	sometimes
			514	severe	No
	gidB	Resistantce		not defined	
Injectables	16S rRNA	Resistance	A1401G	minor	Yes
KAN, AMK		Resistance	C1402A	moderate	Maybe
VIO, CAP		Compensatory	G1484U	compensatory	restores base pairing in rRNA
	TlyA	Compensatory	methylates *rrs* 1402	upregulation *tlyA* partially compensates A1401G fitness loss	
	TlyA	Resistance to CAP & VIO	loss of TlyA function		
FQ	*gyrA*	Resistance	AAs 89, **90,** 91, & **94**	no fitness loss documented	Yes
			Gly88Asp	minor	No
			Gly88Cys	severe	No
		Compensatory	*glgC* mutations	Partial compensation for Gly88Cys	in *M. smegmatis*

Abbreviations: INH, Isoniazid; RIF, Rifampicin; STR, Streptomycin; Kan, Kanamycin; AMK, Amikacin; VIO, viomycin; CAP, Capreomycin; FQ, Fluoroquinolones; comp., compensates. See text for details and references.

inhA promoter lead to the synthesis of more InhA protein than the INH can fully inhibit and thus low-level INH resistance (0.2–1.0 mg/L). The most common promoter mutation is *inhA* C-15T, in which the 15th nucleotide (C) in front of the *fabG1-inhA* operon is replaced by a T, but mutations at −8, −17, −47 have also been described [37,38]. Structural mutations in the InhA binding pocket for the NADH cofactor [39] can also confer resistance. These may function by allowing the acyl-ACP substrate to enter the InhA active site first, thereby excluding INH from entering and forming an inhibitory complex with NADH [40,41]. The most frequent structural mutation substitutes the serine at amino acid 94, but resistance is also conferred by mutations that substitute the isoleucine at amino acid 194 [42], which forms part of the INH/NAD + binding pocket in the tertiary structure of InhA. Fitness costs associated with *inhA* mutations have not been described.

In contrast, the loss of the KatG catalase/peroxidase activity makes the strain less virulent [43], presumably because KatG protects against the oxidative defenses of the host immune system [44] and may also eliminate toxic peroxides generated during bacterial oxidative metabolism [45]. The loss of KatG activity thus increases oxidative stress within the bacteria and decreases survival within the host [46]. The most important mutation is the KatG Ser315Thr substitution, which accounts for about half of all *katG* gene mutations in clinical INH-R isolates [34]. Because it eliminates the enzyme's ability to activate INH while preserving most of its catalase/peroxidase activity [33,47], this particular mutation confers high-level INH-R (1–5 mg/L) while retaining fitness and is found in most highly transmitted MDR and XDR-TB isolates. Although the KatG Ser315Thr and the *inhA* promoter mutations are the most common in INH-R clinical isolates, they are uncommon in INH-R *M. tb* strains selected *in vitro* [48].

Most of the other mutations or deletions in KatG seriously compromise its catalase/peroxidase function and thereby strain virulence, and it has been proposed these can be compensated by increasing the expression of AhpC, an alkylhydroperoxidase [49]. AhpC has no role in conferring INH-R and the AhpC protein is undetectable in DS *M. tb* but can be detected in some INH-R strains with *katG* mutations. Sherman and colleagues found that the increased expression of *ahpC* in these strains was due to mutations in its promoter region [49]. However, although the *katG* mutant strains with upregulating *ahpC* promoter mutations regain peroxidase activity, there has been disagreement about whether they also regain virulence. Wilson et al. [50], using an antisense construct, found that decreasing the expression of *ahpC* reduced the virulence of *katG* mutant INH-R *M. bovis* strains overexpressing *ahpC*, and also reduced the virulence of WT INH sensitive strains. In contrast, Heym et al. [43] found that the virulence of *katG* mutant INH-R strains was not restored with the upregulation of *ahpC*. These discordant results have never been experimentally resolved, but if increased AhpC expression has a compensatory effect, it must be slight, because mutations in the *ahpC* promoter region are found in only 16% of INH-R strains [20] with *katG* mutations other than Ser315Thr [20,51]. Furthermore, *ahpC* promoter mutations are sometimes found in strains with the Ser315Thr katG mutation [52] (3/572 [20]), which preserves most of the KatG activity and therefore shouldn't require a compensatory increase in AhpC activity, and are also occasionally found in INH sensitive strains with intact *katG* genes [20,40].

Highly transmitted strains will infect many people and generate clusters of patients whose isolates have very similar genotypes. A study in the Netherlands found that while many INH-R strains had less clustering than DS strains, those with the Ser315Thr KatG substitution showed no evidence of a reduction in transmission [53]. Similarly, a study in San Francisco found that strains with the Ser315Thr katG mutation were transmitted and found in clusters, but none of the strains with other *katG* mutations, even those with the *ahpC* promoter mutation, caused secondary cases [54]. Further evidence comes from a case control study in Peru [55], where strains with the KatG Ser315Thr substitution had more than twice the odds of generating a secondary case compared to strains with INH-R due to other mutations.

3. Rifampicin

When RIF was introduced in the late 1960's and given in combination with isoniazid, it reduced the duration of TB therapy from two years to just 9 months [56]. The target of RIF is the beta subunit of the DNA dependent RNA polymerase (RNAP), the polymerase that synthesizes RNA from the DNA template. Drug resistance mutations alter this critical enzyme and, not surprisingly, incur a fitness cost [13–17]. Several studies have found that 95–98% of all RIF-R *M. tb* strains have mutations within an 81 bp region termed the Rifampicin Resistance Determining Region, or RRDR of *rpoB* [57,58], the gene encoding the beta subunit of the RNAP. Over a hundred mutations, insertions and deletions in this region have been associated with RIF-R, but nearly 85% involve substitutions in the codons for just three amino acids – 435, 445 and 450, and the most common substitution, Ser450Leu, is found in 40%–50% of all RIF-R strains [58–60]. These are the amino acid numbers in the *M. tb* RpoB protein, but this enzyme is well conserved in many bacteria where mutations conferring RIF-R occur in equivalent amino acids. Therefore, to facilitate comparisons, the numbering system for the comparable amino acids of *Escherichia coli* RpoB is often used. The equivalent of *M. tb* RpoB amino acid 450 is amino acid 531 in *E. coli* [61].

The success of RIF-R strains seems to depend upon the characteristics associated with the particular RIF-R mutations – the level of resistance, the initial fitness cost, and the propensity to accumulate CM to alleviate the fitness costs [18,62]. Relative fitness can be measured as the growth rate of the resistant strain compared to the unmutated, drug sensitive parent, or in growth competition assays with the parent strain [63,64] or by the relative ability to cause disease or death in an animal model. Studies in *Salmonella typhimurium* [65] and *E. coli* [66] found that RIF-R strains were generally avirulent in mice. When allowed to grow in mice or *in vitro* cultures, a few reverted to be RIF-S, but most retained their resistance while regaining virulence, apparently due to the accumulation of secondary compensatory mutations (CM) that were often in the same *rpoB* gene.

Billington [67] studied individual *in vitro* selected RIF-R mutants of *M. tb* and found that strains with the *rpoB* His445Arg substitution grew much slower than the RIF-S parent strain, while those with His445Asp and His445Tyr substitutions grew only moderately slower than the parent strain [67]. In contrast, strains with the Ser450Leu substitution, the mutation most commonly found in RIF-R *M. tb* clinical isolates [68], grew nearly as well as the parent, and one strain with this mutation grew slightly better, presumably because it had acquired an unidentified CM. The most clinically relevant finding in this study was that the *in vitro* selected strains with the mutations most frequent in RIF-R clinical isolates did not demonstrate a dramatic reduction in fitness, implying that there was no selective pressure for the mutations to spontaneously revert to susceptibility even in the absence of drug therapy, and therefore the global prevalence of RIF-R strains would likely increase.

Gagneux et al. [69] selected RIF-R mutants of *M. tb in vitro* and similarly found that different mutations in the RRDR have varying fitness costs. Some, such as the RpoB Arg448Glu substitution, had severe fitness costs, leaving the strain only 60% as fit as the unmutated Rif-S parent strain, while strains with substitutions in amino acid 445 or Ser450Trp were about 80% as fit. As seen by Billington [67], strains with the Ser450Leu substitution were the least compromised, maintaining 90% fitness. They also compared the fitness of clinical *M. tb* strains that developed RIF-R during treatment with isolates of the same strains before treatment. Out of 10 clinical strain pairs examined, five had the Ser450Leu substitution, and four of these had a fitness that was equal or greater than the parent strain, suggesting they had acquired CMs.

Comas et al. [70] reasoned that mutations compensating for the fitness costs of the RIF-R mutations in *rpoB* might occur in other subunits of the polymerase complex. Accordingly, they performed whole genome sequencing of Gagneux's clinical *M. tb* strain pairs and also six *in vitro* selected RIF resistant strains that had been serially sub-cultured over 45 weeks in the absence of RIF [70]. RNAP is made up of 5 subunits: one

beta, encoded by *rpoB*; two alphas, encoded by *rpoA*; one beta-prime encoded by *rpoC*; and one omega, encoded by *rpoZ* [71] Mutations in *rpoA* or *rpoC* were found in one of the six evolved *in vitro* mutants and in three of the four paired clinical strains with fitness equal or better than their Rif-S parent, but in none of the six strains with reduced fitness. They then screened 329 clinical MDR-TB strains from global sources and found that 89/329 (27.1%) had a non-synonymous mutation in *rpoA* or *rpoC,* and all of these strains also had a mutation in the RRDR. Putative CM in *rpoA* or *rpoC* were found in 19.7% of all global MDR strains, but in 31.3% of MDR strains from high MDR-TB burden countries, consistent with the success of the MDR strains in these countries. None of the 11 RIF-R strains without RRDR mutations had mutations in *rpoA* or *rpoC,* nor did any of the 171 RIF-S strains [20].

Studies in *Salmonella enterica* [72] showed that strains with the Ser531Leu mutation (Ser450Leu in *M. tb*) had a fitness cost of 27%, but after 280 generations in culture, daughter strains that had evolved normal growth contained a variety of mutations in *rpoA, B or C.* In RIF-R strains of *Pseudomonas aeruginosa* [73], only those with mutations at RpoB amino acid 531 accumulated CM that restored fitness. Different substitutions for serine 531 had distinct rates of developing CM, obtained different levels of fitness and had different compensatory amino acid substitutions within RpoB.

4. The structural basis of Rif resistance and compensatory mutations

The structure of the DNA dependent RNA Polymerase (RNAP) contains a "crab claw" motif with the upper pincer arm formed by the beta subunit, encoded by *rpoB*, and the lower by the beta prime (β′) subunit, encoded by *rpoC* [74]. The RIF binding site is within the beta subunit, close to the site of RNA synthesis on the surface of the deep cleft of the claw, which is the exit tunnel for newly synthesized RNA [75,76]. By binding to RpoB, RIF blocks the elongation of RNA when the transcripts are just 2–3 nucleotides long [77,78]. The CM occur predominantly at the interface of the alpha and beta prime subunits (encoded by *rpoA* and *rpoB*, respectively) near the catalytic site of the RNAP. Three fourths of all CM are in strains with Ser450Leu. Of these, about 9% are in RpoA [75], but the majority are in RpoC, most commonly RpoC Val483Gly Fig. 1) [70,79,80]. There are also some *M. tb* genotypes that have CM in RpoB outside of the RRDR [20].

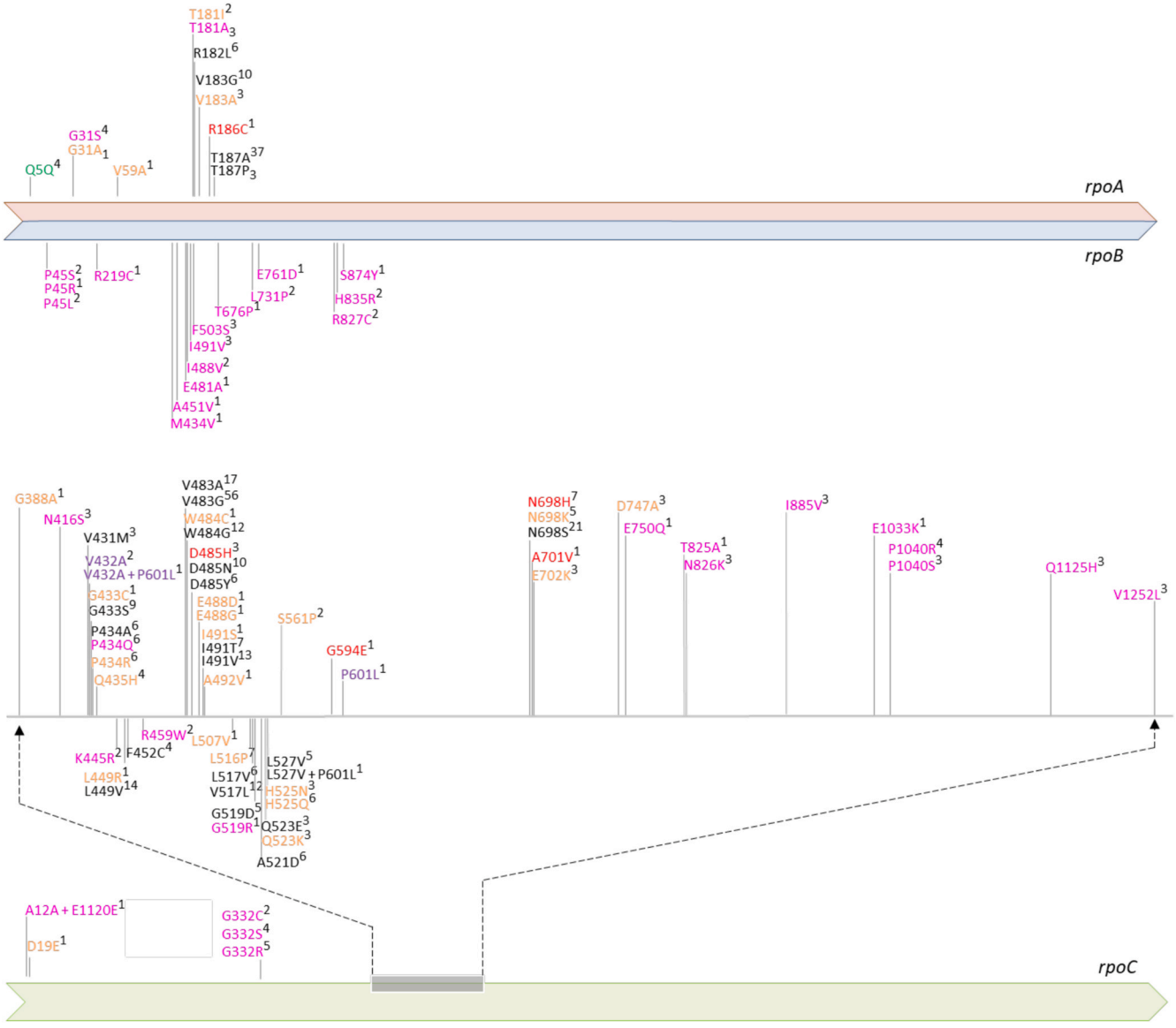

Fig. 1. Putative Compensatory mutations in RIF-R strains of *M. tuberculosis*. Superscripts refer to the total number of strains with each mutation described in the relevant references. [20,70,78,79,103–109].

Stefan et al. [75] looked at functional alterations in RNAP enzymes containing the three most common RIF resistance mutations in RpoB - Ser450Leu, Asp435Val and His445Tyr, and then each of these together with either of the two most frequent CM in RpoC – Val483Gly and Phe452Leu. Mutations conferring RIF-R reduce RIF binding, but also impede the exit of newly synthesized RNA, and the CM seem to somehow compensate by facilitating RNA exit [62]. Overall, the functional effects of the RRDR *rpoB* mutations are complex, but the most common *rpoB* mutations perhaps have a favorable balance between stability, elongation rate and termination efficiency, and also show a propensity to have their fitness defects corrected by CM [18]. The RpoB Ser450Leu mutation not only has the least effect on RNAP function, but also the highest frequency of CM that allow RpoB function to be almost completely restored. The structural basis for the advantages with this mutation has not been explained.

5. Streptomycin

Studies with *Salmonella typhimurium* [65] and *E. coli* [81], found that some mutations conferring streptomycin resistance (STR-R) have a fitness cost and reduced virulence. Although STR is no longer a mainstay of tuberculosis treatment, it is included here because the mechanism to compensate for some STR-R mutations is interesting and novel. Streptomycin interacts with the ribosome to decrease translational accuracy, resulting in mistranslated proteins that are nonfunctional and toxic for the bacteria. In clinical *M. tb* strains, most resistance mutations are found in *rpsL*, which encodes protein S12 of the 30S ribosomal subunit. Some resistance mutations, termed "restrictive", counteract the effect of streptomycin by conferring a hyperaccuracy, but this comes with a fitness cost due to reduced translation and slower growth rates [65]. A few resistance mutations are so stringent that they become dependent upon the presence of streptomycin to reduce the accuracy enough to allow them to grow [82]. The compensatory mutations that reduce the translational accuracy and restore the fitness of strains with restrictive mutations are found at other sites in *rpsL* or within *rpsD* or *rpsE*, which encode ribosomal proteins S4 and S5 respectively [65].

Mutations that do not affect the growth rate are termed "non-restrictive" and have a minimal fitness cost. The most common STR-R mutations in *M. tb* affect RpsL amino acids 43 or 88. On *in vitro* selection, mutations replacing the lysine in amino acid 43 with the non-restrictive arginine, or the restrictive, fitness-reducing threonine or asparagine substitutions [83], occur with similar frequencies. In STR-R clinical *M. tb* isolates however, >98% of strains with mutations in RpsL amino acid 43 have the non-restrictive arginine substitution [83,84].

STR-R mutations can also occur in the 16S ribosomal RNA [85–87]. An *in vitro* selection for Str-R strains of *M. smegmatis* found mutations in 16S rRNA nucleotides 522, 523, and 526, which have relatively slight fitness costs, or in nucleotide 524, which has a much greater fitness cost. Although the mutation in 524 was the most common with *in vitro* selection, it is the only one that is not found in Str-R clinical *M. tb* strains, where the equivalent nucleotides are 512, 513, 514 and 516 [83]. Therefore, although it is possible that low fitness STR-R strains can accumulate CM, they have little clinical relevance because the mutations that seriously compromise fitness and require CM are rarely seen in clinical *M. tb* isolates. This could be because these strains don't survive long enough *in vivo* to develop CM, or because the CM do not sufficiently restore fitness to allow them to survive at all *in vivo*. Mutations in the methyltransferase GidB also confer STR-R, and may have a fitness cost [88], but this has not been well studied.

6. Other injectable antibiotics

The mutations that confer resistance to the injectable antibiotics – the aminoglycosides kanamycin and amikacin and the tuberactinomycin antibiotics viomycin and capreomycin – occur mostly in the 16S rRNA, and several different mutations can be selected *in vitro*. Mutations with the least fitness cost predominate in resistant clinical *M. tb* strains, particularly the replacement of adenine for guanine at nucleotide 1401 (A1401G), which corresponds to 1408 in *E. coli* [89,90]. Some of the less fit strains have 16S rRNA mutations that disrupt base pairing in the rRNA secondary structure, although the base pairing can be restored with CM, and some 16S rRNA mutations and CM pairs i.e., 1402 C→A/1484 G→U, are found in aminoglycoside-resistant *M. tb* clinical isolates. Promoter mutations that increase the expression of *eis*, which encodes an aminoglycoside acetyltransferase, confer low-level kanamycin resistance but have no detectable fitness costs [91].

There is an interesting compensatory mechanism associated with the 16S rRNA A1401G resistance mutation [92]. Mutations that eliminate the function of the rRNA methylase TlyA confer resistance to capreomycin and viomycin, presumably because the binding of these antibiotics to the 16S rRNA requires methylated nucleotides. The A1401G mutation reduces the fitness of the bacteria by about 10%, but somehow causes increased expression of TlyA, which methylates the adjacent nucleotide 1402. The methylation of 1402 partially compensates for the loss of fitness incurred with the A1401G mutation but reduces the resistance to capreomycin and viomycin. This compensatory mechanism is not the result of a mutation but rather a poorly understood upregulation of TlyA. In the presence of capreomycin, however, the expression of TlyA is somehow reduced. This results in less methylation of nucleotide 1402 and thereby increased resistance to capreomycin and viomycin, but also less compensation for the fitness cost of the 1401 mutation. Mutants with no TlyA function are resistant to capreomycin and viomycin but cannot compensate for the decreased fitness of the A1401G mutation. No clinical strain has been described with both the A1401G mutation and a TlyA inactivating mutation, presumably because the uncompensated fitness cost of the 1401 mutation would not allow the strain to survive in a host [92–94].

7. Gyrase mutations and fluoroquinolone resistance (FQ-R)

There is a suggestion that some pre-XDR and XDR-TB strains are not well transmitted, perhaps due to the FQ-R mutations [20]. Although globally, the FQs are amongst the most widely used antibiotics and FQ-R is a problem in many bacteria [95], there are few reports on the fitness costs of FQ-R mutations. The targets of the FQs are the type II topoisomerases – DNA gyrase and the Topoisomerase IV (Topo IV), which is similar to the DNA gyrase but not present in mycobacteria. In *M. tb*, the FQ-R mutations are principally in *gyrA,* the gene encoding the gyrase A subunit [96], with a few in *gyrB*, which encodes the gyrase B subunit.

A study in *Salmonella typhimurium* found that a strain with a *gyrA* mutation conferring resistance to nalidixic acid had a slower *in vitro* growth rate and was outcompeted by a WT strain in a mouse infection [65]. After being passaged through mice, however, the WT growth rate was restored through the acquisition of an unidentified CM mapping close to *gyrA*. In *Streptococcus pneumoniae* some specific combinations of ParC (the A subunit of Topo IV) and GyrA substitutions had a fitness cost, while other combinations made the strain more fit than the FQ-S parent [97]. Luo [98] found that FQ-R strains of *Campylobacter jejuni* with *gyrA* mutations outcompeted FQ-S strains in chickens, although the effect depended upon the genetic background of the strain.

In both *M. smegmatis* [99] and *M. aurum* [100], the most common *in vitro* selected FQ-R mutations replace the aspartate in GyrA amino acid 94 and show minimal fitness costs. Some strains with the Asp94Gly substitution actually grew better than the FQ-S WT parents. Mutations at amino acid 94 are also the most common in FQ-R clinical isolates of *M. tb*. In contrast, strains with the Gly88Asp mutation grew slightly slower than WT, and a strain with the Gly88Cys substitution grew much slower, likely explaining why these are mutations are infrequently recovered with *in vitro* selections and rarely seen in clinical isolates. When the Gly88Cys strain was exposed to a higher level of levofloxacin it acquired an additional Gly94Asn mutation and grew nearly as well as

the WT parent. When Gly88Cys strains were allowed to evolve over 15 passages *in vitro* without antibiotics, their fitness increased and genome sequencing revealed that they had acquired mutations in *glgC*, which encodes an ADP-glucose pyrophosphorylase. This putative CM suggests that the fitness of the resistant strains was restored through an alteration in the bacterial energy metabolism [100].

Most combinations of *rpoB* and *gyrA* mutations, as occur in XDR-TB, were found to show a slightly additive loss of fitness, but strains with an amino acid change in RpoB 445 together with either the GyrA Asp94Asn or Asp94Gly substitutions grew better than strains with either single mutation. When strains of *M. smegmatis* contain two gyrase mutations, they tend to have high level FQ resistance but slightly reduced fitness [101]. The overall impression is that the fitness of a FQ-R *M. tb* strain depends upon an interplay between the genetic background of the strain, the specific *gyrA* mutation and the mutations conferring resistance to other drugs [99].

A recent study demonstrated how resistance mutations and their fitness costs can be modulated by the strain genotype [102]. Different strains of *M. tb* accumulate FQ-R gyrase mutations *in vitro* at different frequencies, with different preferences for the specific mutations and different fitness costs. Clinical strains showed lineage specific mutation preferences: FQ-R strains of lineage 2 predominantly had the GyrA Asp94Gly substitution, while lineage 4 strains more commonly had the GyrA Ala90Val substitution. Most of the studies on resistance, fitness and compensatory mutations cited in this review were performed *in vitro* using a single mycobacterial strain, but the acquisition and effects of these mutations are likely to be more complex and variable when they occur in the variety of *M. tb* strains that cause tuberculosis disease [22].

Author contributions

Amel Kevin Alame Emane: Conceptualization; Formal analysis; Writing original draft

Xujun Guo: Funding acquisition; Supervision; Project administration.

Howard E. Takiff: Conceptualization; Formal analysis; Writing original draft, review & editing.

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