

Diversity and evolution of drug resistance mechanisms in *Mycobacterium tuberculosis*

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Abstract: Despite the efficacy of antibiotics to protect humankind against many deadly pathogens, such as *Mycobacterium tuberculosis*, nothing can prevent the emergence of drug-resistant strains. Several mechanisms facilitate drug resistance in *M. tuberculosis* including compensatory evolution, epistasis, clonal interference, cell wall integrity, efflux pumps, and target mimicry. In this study, we present recent findings relevant to these mechanisms, which can enable the discovery of new drug targets and subsequent development of novel drugs for treatment of drug-resistant *M. tuberculosis*.

Keywords: *Mycobacterium tuberculosis*, antibiotic resistance, compensatory evolution, epistasis, efflux pumps, fitness cost

Introduction

Tuberculosis (TB) is a significant public health concern with a high disease burden and mortality rate.¹ The disease is caused by members of the *Mycobacterium tuberculosis* complex (MTBC), a group of closely related human-adapted (*M. tuberculosis* [MTB] and *Mycobacterium africanum*) and animal-adapted (*Mycobacterium bovis*, *Mycobacterium mungi*, *Mycobacterium pinnipedii*, *Mycobacterium microti*, and *Mycobacterium caprae*) strains, as well as smooth tuberculosis bacilli (*Mycobacterium canettii*).² Although MTBC species have a remarkable range of mammalian hosts and morphologies, their genomes exhibit $\geq 99\%$ homology, providing evidence that they evolved from a single ancestor in Africa 70,000 years ago.²⁻⁴ However, following advances in agriculture and animal domestication, MTB emerged as a human pathogen that has caused millions of deaths and continues to threaten human health globally.^{5,6} Successful control and prevention of MTB infection requires tools for a rapid and accurate diagnosis, as well as strategies for effective treatment. Current antibiotics used to treat MTB infection are isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB).^{7,8} Misuse or misadministration of drugs can facilitate the emergence of drug-resistant strains via compensatory evolution, epistasis, and clonal interference phenomena that modulate MTB fitness. These various mechanisms of adaptation have led to the evolution of different drug-resistant levels of MTB strains, including multidrug resistant (MDR; resistance to INH and RIF), extensively drug resistant (XDR; resistance to fluoroquinolones [FQs] and one of the injectable aminoglycosides [AGs]), and totally drug resistant (TDR; resistance to all known drugs).^{9,10}

Therefore, the aim of this review was to highlight recent findings related to mechanisms implicated in the emergence of MTB drug resistance and outline some possible

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drug targets to contribute to efforts aimed at discovering novel TB treatment.

Development of drug-resistant MTB

MTB diversity and drug resistance

Genomic comparisons have increased our understanding of MTBC diversity. For example, analyses of single nucleotide polymorphisms (SNPs) and the presence or absence of deletion regions (MTB-deleted region 1 or regions of difference) within MTBC genomes have identified several lineages and sub-lineages with distinct characteristics and distributions.² For instance, lineage 2 (East Asian) and 4 (Euro-American) strains are widely distributed, while lineage 3 (Central Asian and East African Indian) strains are restricted to particular regions in Asia and Africa. These three lineages are characterized by deleted regions and are denoted as “modern lineages.” In contrast, lineage 1 (Indo-Oceanic) strains are common in the Indian Ocean region and the Philippines. This lineage, together with lineages 5 and 6 (West African), and animal lineages comprise the “ancient lineages” and have no deletions characteristic of the modern lineages.^{2,11} Finally, lineage 7 is considered as an intermediate lineage and has recently reported in Ethiopia.¹²

Genomic differences among MTBC lineages impact their capability to cause disease and develop drug resistance. For instance, members of the modern lineages are associated with greater disease burden and drug resistance than the ancient lineages, likely due to a high rate of accumulating spontaneous mutations during replication.^{13,14} Epidemiological studies have illustrated that lineage 2 strains have a higher rate of developing resistance ranging from 1.6×10^{-5} to 5.4×10^{-3} than lineage 1.¹⁵

Despite the accumulation of spontaneous mutations among MTBC lineages, their specific (and perhaps unique) drug resistance mechanisms remain unknown. However, increased mutations in DNA repair system and SOS response genes within lineage 2 genomes enhance the possibility of these strains developing resistance and generating mutator phenotype.^{14,16} Nonetheless, these findings cannot sufficiently explain several enigmas in these strains, such as how the same mutation among MTBC can generate a different level of resistance. Moreover, how do some strains tolerate certain mutations better than others, and why are some strains more strongly associated with infectious transmission and outbreaks? These questions could be resolved by studies of compensatory evolution, epistasis, and clonal interference, as these mechanisms could have large impact on mutation rate acceleration and MTB fitness modulation.^{17–21}

Compensatory evolution and MTB drug resistance

Drug concentration is a primary determinant of resistance-associated mutations during drug therapy.²² Mutations develop when the drug concentration is not optimal, although mutations impose a fitness cost on bacteria that targets genes encoding essential biological functions, often leading to reduced bacterial growth, survival, and virulence.^{23,24} Contrary to fitness cost concept, several studies have documented that some frequently transmitted MTB strains undergo low- or no-cost mutations but exhibit high level of resistance to drugs.^{17,19,20} Thus, these strains may harbor resistance mechanisms developed through compensatory evolution, which can modulate MTB fitness (Figure 1).^{25,26}

Compensatory evolution is mediated by the acquiring of a second mutation that minimizes the deleterious effect (resistance cost) of the original mutation. This mechanism allows MTB to increase its fitness without losing the resistance phenotype.²⁷ Compensatory evolution can develop from either additional or alternative mutations that occur in intra- or extragenic loci.²⁸

In MTB, compensatory evolution associated with INH resistance occurs when a mutation in the regulatory region of *ahpC* leads to overexpression of alkyl hydroperoxide reductase (AhpC), which may compensate for the fitness cost of Ser315Thr mutation in *katG*, normally encodes a catalase-peroxidase, converting INH into a bioactive form.²⁹

Compensatory evolution of RIF resistance has also been reported.^{30,31} In one study, mutations in *rpoB*, which encodes the β subunit of RNA polymerase, were detected in 95% of clinical isolates and conferred a high level of RIF resistance, but have also been associated with noticeable fitness cost.^{17,30,32} However, S531L mutation was seen in most MDR isolates that exhibited a no- or low-cost fitness effect. This phenomenon is explained by the acquisition of compensatory mutations in neighboring *rpoA* and *rpoC* genes, which can mitigate the fitness cost of S531L.^{33–36} Comas et al found that up to 30% of MDR cases in high MTB burden countries carried mutations in *rpoA* and *rpoC*, suggesting they may play a role in spreading MDR strains in these countries.³³ Interestingly, a recent study revealed the compensatory role of an intragenic V615M mutation, located in *rpoB* gene, with respect to RIF resistance-associated *rpoB* mutations. The study showed that V615M mutation can modulate RNA polymerase bridge helix structure and contribute to an increased rate of transcription elongation, thus compensating for defective RNA polymerase activity associated with S531L mutation.³⁷

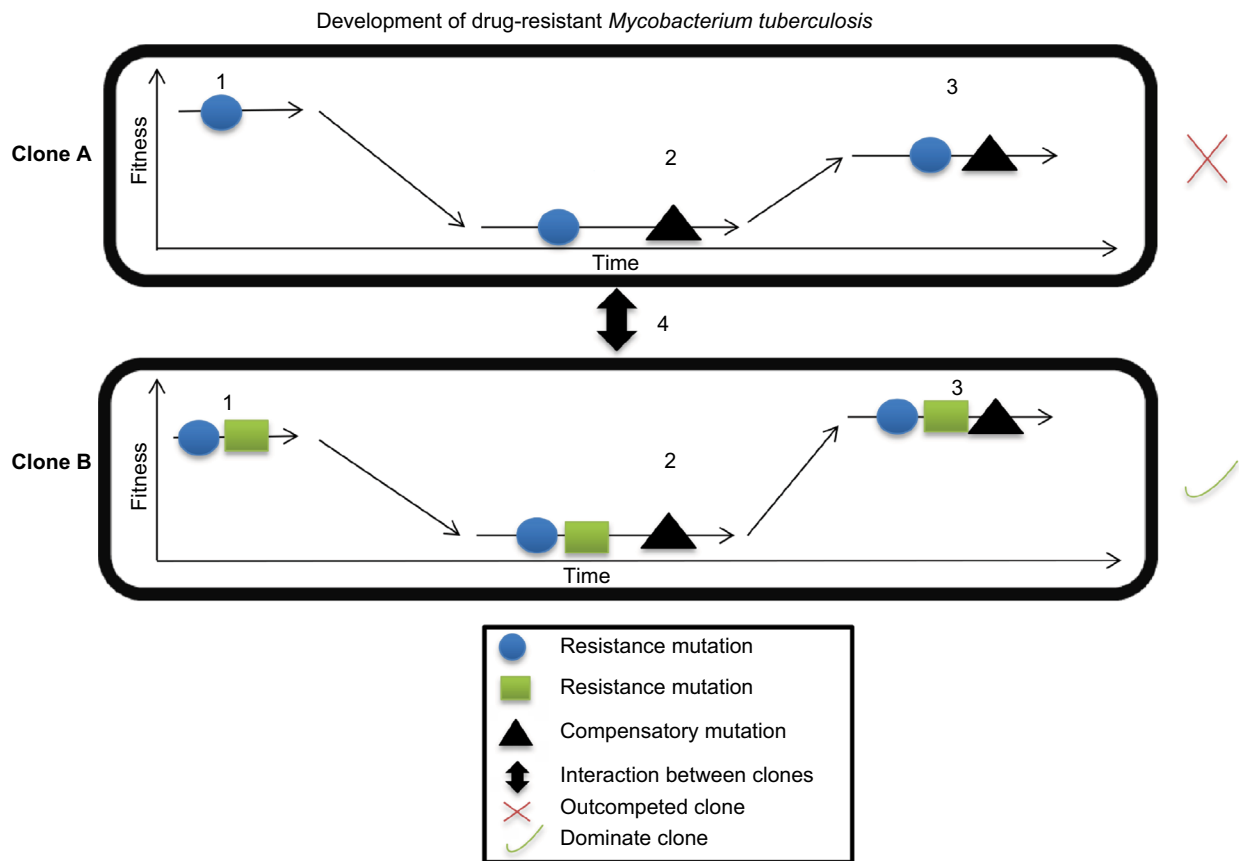


Figure 1 Development of drug resistance.

Notes: 1, when MTB acquires mutations during therapy, they reduce MTB fitness (clones A and B). 2, an acquired secondary mutation restores MTB fitness. 3, the epistatic interaction between mutations improves MTB fitness and maintains drug resistance within the specific MTB genetic background. 4, clonal interference determines clone fate via competition, which leads to emergence of the most dominate clone with drug resistance in the population and elimination of the clone with a lower mutational effect.

Abbreviation: MTB, *Mycobacterium tuberculosis*.

In addition to compensatory mutations, alternative mechanisms of fitness compensation may exist. For example, Freihofer et al³⁸ found that altered gene regulation can also reduce the deleterious effects of certain genetic mutations. The study found that emergence of the A1408G mutation in the 16S rRNA gene is accompanied by overexpression of *thyA*, which encodes a methyltransferase, resulting in methylation of neighboring 16S rRNA position C1409 and increased MTB fitness. The identification of non-mutational mechanisms could provide a new strategy for optimizing current treatment regimens through inhibition of the compensatory event, leading to disruption of the stabilization of drug resistance transmission.

Role of epistasis and genetic background in MTB drug resistance

Epistasis occurs when several mutations interact with each other to express new advantageous traits for an organism and are often necessary for bacteria to modify their fitness cost.³⁹

During epistatic interactions, the effect of multiple mutations is greater or less than the effect of the individual mutation and can lead to either beneficial or deleterious phenotypes.⁷ Thus, epistasis is classified as 1) positive (antagonistic), 2) negative (synergistic), or 3) sign.⁴⁰

In positive epistasis, the net fitness of the interactions is higher than expected (Figure 2).⁸³ A study has reported the role of positive epistasis in drug resistance development in MTB. Borrell et al⁴¹ identified a particular combination of mutations in *rpoB* and *gyrA* that conferred resistance to RIF and ofloxacin (OFX). The study showed that a *gyrA* D94G mutation is associated with improving deleterious fitness effects. Thus, *gyrA* D94G is correlated with positive epistasis in MTB, and it is frequently occurred within XDR strains.

Contrary to positive epistasis, negative epistasis is characterized by fitness lower than expected after mutation interactions. In bacteria, interactions between beneficial mutations provide a simple additive effect on fitness, while interactions between deleterious mutations are lethal (Fig-

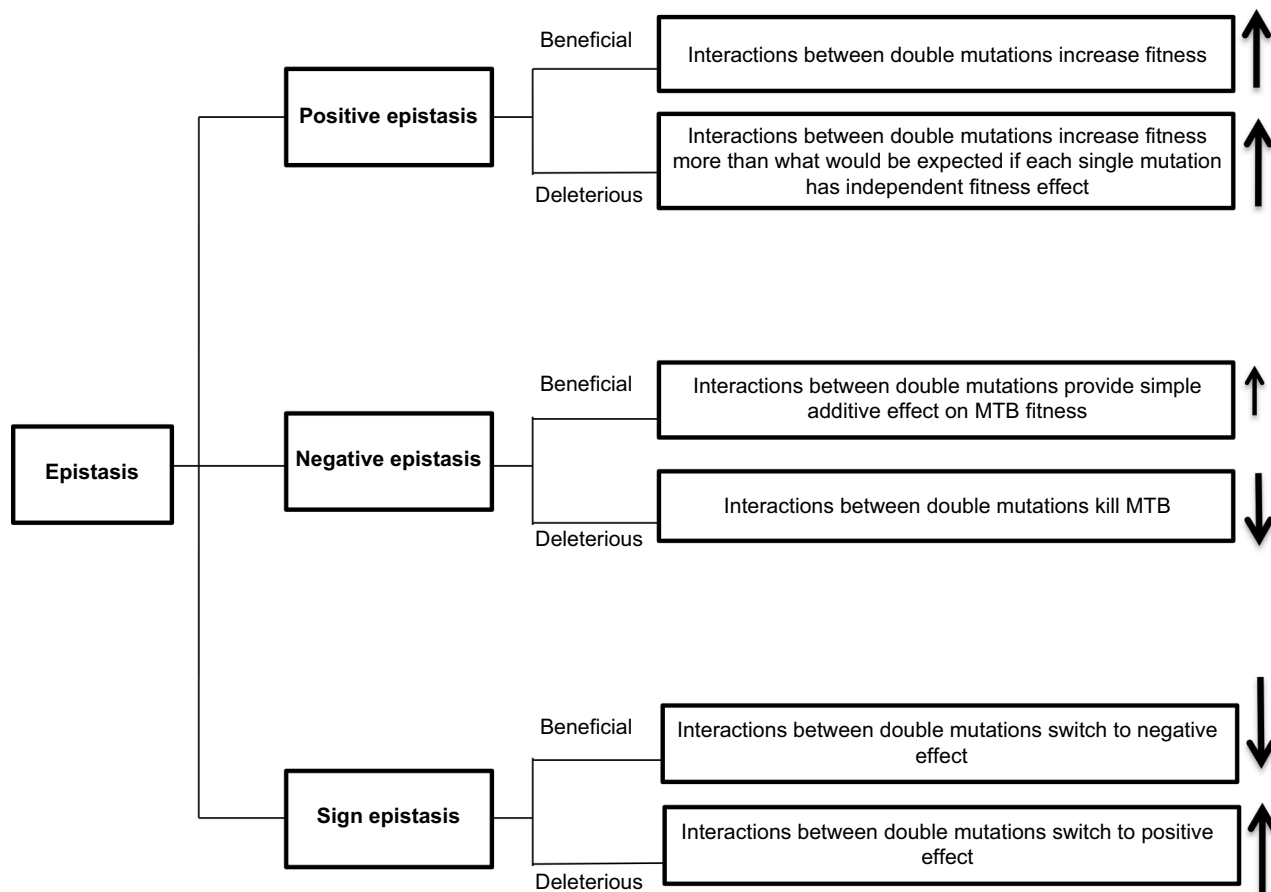


Figure 2 Forms of epistatic interaction between mutations.
Note: (↑) indicated high MTB fitness and (↓) indicated low MTB fitness.
Abbreviation: MTB, *Mycobacterium tuberculosis*.

ure 2).⁸³ However, in sign epistasis, the fitness of mutations depends on the genetic background of the bacteria. These mutations can be deleterious, beneficial, or neutral. Reciprocal sign epistasis is an extreme form of the interactions in which beneficial mutations together exert a negative effect or when deleterious mutations become positive (Figure 2).^{40,42} The interaction between compensatory and drug resistance mutations is an example of sign epistasis.⁴³ When compensatory mutations occur in a susceptible genetic background, they become deleterious. Thus, the acquisition of resistance mutations promotes the epistatic interaction between compensatory and acquired resistance mutations, which results in the emergence and maintenance of the resistance phenotype in that particular bacterial genetic background.⁴⁴

In addition, increasing evidence supports the role of sign epistasis in MTBC diversity.⁴³ This is mainly due to the epistatic interactions between the mutations within the MTBC genetic background, the acquired resistance mutations, and the compensatory mutations.⁴⁵ Fenner et al⁴⁶ found that a

mutation in either *katG* or *inhA* confers different levels of resistance. Lineage 2 strains carry mutations in both *katG* (high INH resistance) and *inhA* (low INH resistance) genes and show different levels of drug resistance compared to lineage 1 bacteria, in which only the *inhA* mutation has been identified. Similarly, when bacteria from different lineages were exposed to the same dose of RIF, they exhibited different fitness costs and resistance levels.¹⁷ These data support the role of epistasis interactions between MTBC genetic background and acquired mutations that confer various levels of resistance across MTBC lineages.

Dynamics of clonal interference in MTB

Depending on the bacterial population size, mutation rate (U), and distribution of fitness effects, various mutations can simultaneously develop in a single population.⁴⁷ In this situation, clonal interference may occur and significantly impact resistance development in the population. When two distinct resistance mutations develop independently within distinctive

bacterial individuals, they compete with each other. Thus, a clone with a greater mutation effect outcompetes a clone with smaller mutation effects, which is then eliminated from the population (Figure 1).⁴⁸

The intra-host evolution of MTB provides a straightforward model for understanding clonal interference in vivo. Some studies have reported competition between MTB clones in a single patient sample. Sun et al⁴⁷ examined seven isolates from three patients; the first patient was free from MTB drug resistance, but after 19 months of treatment, four independent mutations were detected: three mutations in *katG* and one mutation in the regulatory region of *inhA*. After 5 months, most of the mutations reverted, and only one mutation in *katG* was detected. The second patient harbored MTB with a mutation in *rpoB* (L533P) but was still sensitive to RIF. After 18 months, the L533P mutation was replaced with a second mutation in *rpoB* (H526Y), leading to RIF-resistant MTB. The third patient was a relapsed case of MTB with two unfixed mutations of *ethA* (L35R and A341E) after 11 months of treatment that showed no change in EMB resistance status. These observations illustrate how the competition and interchange between resistance-related mutations can lead to MDR. Similarly, Eldholm et al⁴⁹ followed an XDR-TB patient for 3.5 years and performed genome sequencing of nine isolates from the same patient. They observed a high level of heterogeneity in the isolate population: 35 mutations were identified, including 20 transient and 15 fixed mutations. Eventually, 12 mutations were determined to be drug resistance related, although only seven of these mutations reached fixation stage. This observation indicates that the competition between high and low effective resistance mutations lead to high resistance.

Alternative mechanisms implicated in drug resistance

MTB shows intrinsic resistance to different drugs through various mechanisms, including cell wall or membrane impermeability and efflux pump action. Mutations can enhance intrinsic resistance, generate new proteins that inactivate the drug or block interactions with its target, or alter the target to prevent its recognition by the drugs (Figure 3).⁵⁰

Impermeability of the MTB cell wall and drug resistance

The MTB cell wall structure is unique due to mycolyl-arabinogalactan-peptidoglycan complexes and free glycolipids (e.g., trehalose dimycolate, PPE family proteins, and

phthiocerol dimycocerosates), forming a hydrophobic barrier that limits the entrance of various drugs.⁵¹ Several studies have identified the essential genes, enzymatic activities, and cellular components that increase resistance levels by decreasing cell wall permeability. For example, the expression of monooxygenase (*mymA*) operon is regulated by *virS*, which maintains mycolic acid composition and enhances cell wall integrity.⁵² Mutations in *virS-mymA* lead to increased cell wall permeability and diffusion of INH, RIF, PZA, and ciprofloxacin inside the cells.^{53–56}

The PE11 protein of MTB plays a role in cell wall maintenance and is a putative lipase/esterase involved in cell wall remodeling.⁵⁷ Expression of PE11 in *M. smegmatis* mc²155 modulates cell wall morphology, composition, aggregation, and pellicle formation and mediates resistance to INH, RIF, EMB, vancomycin, and ampicillin. These findings suggest that upregulation of PE11 in MTB reduces penetration of antibiotics during active TB.⁵⁸

Moreover, MTB can preserve cell wall integrity through acquired mutations that lead to overexpression of genes encoding enzymes involved in cell wall synthesis. For example, pro-drugs, INH and ethionamide, which share a similar mechanism of action, are converted into their bioactive forms (isonicotinic-acyl radicals and 2-ethyl-4-amidopyridine, respectively) by *katG* and *ethA* gene products. These bioactive forms react with nicotinamide adenine dinucleotide to form nicotinamide adenine dinucleotide adducts that bind to enoyl-acyl carrier protein reductase (encoded by *inhA*), a key enzyme involved in fatty acid synthase II system, and inhibit mycolic acid synthesis.^{50,59} Therefore, mutations in *inhA* prevent binding of INH and ethionamide to their targets and confer resistance to these drugs.⁶⁰ Furthermore, arabinosyl transferases (which link peptidoglycan with an outer mycolic acid layer to form the mycolyl-arabinogalactan-peptidoglycan complex) may be a target for EMB, a drug that inhibits arabinosyl transferase activity and causes increased cell wall permeability.⁶¹ When *emb* genes acquire mutations, overexpression of *emb* genes and increased EMB proteins can overcome certain levels of the drug. A specific mutation in *embB* at codon 306 may correlate with EMB resistance, and mutations in cell wall synthesis-associated genes *aftA* and *ubiA* lead to overexpression of *embC* and EmbCAB substrates and subsequent resistance to EMB.^{62–64} In addition, novel mutations at *emBA* G43C and G554N and at *embB* S412P have found to confer a high level of resistance to EMB among MTB isolates.^{65,66}

Overall, these findings highlight the role of MTB cell wall maintenance in intrinsic and acquired drug resistance. Thus,

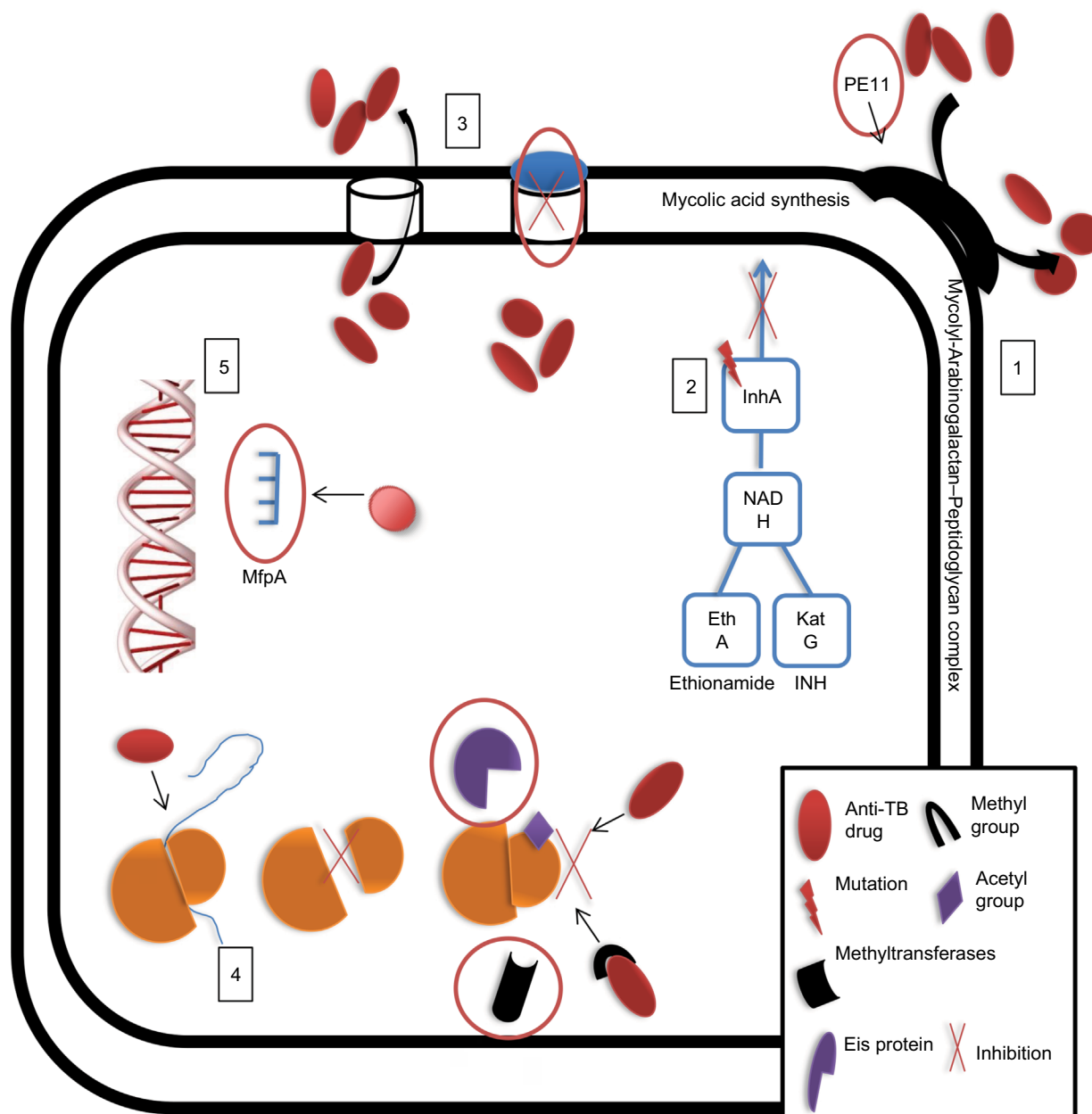


Figure 3 MTB can exhibit resistance to drugs via: 1, intrinsically decreased permeability of the cell wall; 2, acquisition of mutations that block drug entry; 3, extrusion drugs via efflux pumps; 4, modification of the drug or its target, or 5, target mimicry.

Note: Possible drug targets are indicated by red circles.

Abbreviations: INH, isoniazid; MTB, *Mycobacterium tuberculosis*; TB, tuberculosis.

identification of novel proteins (e.g., PE11) that increase the cell wall integrity could facilitate their use as promising drug targets for MTB treatment.

Action of efflux pumps and drug resistance

Efflux pumps are natural drug barriers widely distributed in both prokaryotic and eukaryotic cell walls. These pumps maintain cellular hemostasis and regulate exchange of nutrients across the cell membrane.⁶⁷ They are classified into six

major families based on their energy source, size, and substrates. These include the ATP-binding cassette (ABC), small multidrug resistance (SMR), resistance nodulation division (RND), major facilitator superfamily (MFS), multidrug toxic compound extrusion (MATE), and drug metabolite transporter (DMT) superfamily.⁶⁸ All but except MATE and DMT efflux pumps are specific to MTB strains.⁶⁹ Drug resistance mediated by efflux pumps depends on their basal expression and by drug-induced gene expression or overexpression that result from acquired mutations.⁷⁰

Recently, several studies have used whole-genome sequencing to identify relevant mutations in efflux pump-associated genes that confer resistance. Li et al⁷⁰ examined the expression level of efflux pump genes within MDR isolates and found that at least one efflux pump was overexpressed in eight out of nine isolates, suggesting that this system contributed to the development of resistance to multiple drugs. Interestingly, one MDR isolate carried a mutation in *rpoB* that conferred RIF resistance but intact *katG*, *inhA*, and *oxyR-ahpC*, indicating that efflux pumps, rather than mutations alone, were responsible for the INH resistance. An additional study showed that mutations within the *Rv0678*-encoded transcription repressor of MmpL5 (RND family) led to overexpression of the MmpL5 pump and resistance to clofazimine.⁷¹ Similarly, Kanji et al^{72,73} examined SNPs associated with *Rv2688c*, *Rv0194*, *Rv2936* (*drxA*), *Rv2937* (*drxB*), and *Rv1634*, which encode pumps from the ABC and MFS transporter families, within XDR strains. The study showed significant levels of gene expression compared to susceptible and H37Rv strains, thereby demonstrating the importance of efflux pumps in drug resistance development in XDR isolates. Another study explored the expression of *Rv2686c*, *Rv2687c*, *Rv2688c*, *Rv0933c*, and *Rv1258c* within MDR and XDR isolates and found high expression of the *Rv0933c*-encoded PstB pump (ABC transporter family) in response to FQ treatment, suggesting that the expression of *pstB* is associated with resistance to FQ. In addition, MFS Tap efflux pumps showed a high level of expression, suggesting a correlation with kanamycin resistance.⁷⁴ Expression of *Rv1258c*-encoded Tap pumps is regulated by transcription activator WhiB7, which is more highly expressed following a point mutation in the 5' untranslated region of *whiB7*.^{75–78}

These findings confirm that efflux pumps strongly contribute to developing drug resistance in MTB, thus highlighting efflux pumps as potential candidate targets for novel MTB drugs. Diverse synthetic and plant-derived molecules that act as efflux pump inhibitors (EPIs) have recently been identified, all of which exert different levels of efflux pump inhibition in MTB.⁷⁹ When these inhibitors bind to efflux pumps, they increase drug retention inside the cytoplasm, restore the drug's activity, and prevent the selection of resistant mutants.⁷⁹ These results occur when EPIs act as a single drug such as an SQ109 inhibitor, or enhances the efficacy of certain drug combinations such as timcodar.^{80,81} More recently, Kumar et al⁸² found that synthesis and design of hybrid EPIs by fusion of VerapamilTM with phenothiazines

enhances inhibition of efflux pumps and may enable identification of novel molecules with a high efficacy for killing MTB.

Modification of drugs and their respective targets

An additional mechanism implicated in MTB drug resistance is the modification of either the drug or its target through specific enzymes. These enzymes are often enable acetylation or methylation of the drug or its target to prevent recognition and interaction between the two. One study found that MTB and *M. bovis* carry *erm37*, which encodes an rRNA methyltransferase that blocks interactions between macrolides and the ribosome.⁸³ MTB also expresses enhanced intracellular survival (EIS) proteins, which are homologs of AG acetyltransferases.⁸⁴ These proteins can acetylate multiple sites of AGs, resulting in their inactivation.⁸⁵ Houghton et al⁸⁷ found that EIS proteins can also protect MTB against capreomycin.⁸⁶ A more recent study conducted by Warriar et al determined that MTB can methylate drugs via *N*-methylation, such as inactivation of the “14” drug at the N-5 position via a methyltransferase encoded by *Rv0560c*.⁹³

Target mimicry and drug resistance

Target mimicry is a novel mechanism developed by MDR strains to detoxify FQ drugs, which target DNA gyrase. When FQs bind to DNA gyrase, they inhibit DNA replication, repair, and transcription.⁸⁸ A previous study showed that FQ resistance develops through the *Mycobacteria* FQ resistance protein A (MfpA).⁸⁹ MfpA can resemble the shape, size, and surface of the DNA double helix, suggesting that MfpA mimics the structure of MTB DNA.^{90,91} Once MfpA binds to DNA gyrase, the protein prevents FQ binding and protects MTB from the drug's action.⁹²

Conclusion

The ongoing evolution of resistance mechanisms among MTB population is a serious concern. In this review, we highlighted the major mechanisms that lead to drug resistance. These mechanisms include compensatory evolution, epistasis, clonal interference, decreased cell wall permeability, overexpression of efflux pumps, drug/target modification, and target mimicry. These mechanisms allow MTB to modulate their fitness, enhance their transmissibility, and stabilize the resistance phenotype within their population. Understanding of these mechanisms enable researchers to identify novel drug targets in order to develop effective drugs.

Disclosure

The authors report no conflicts of interest in this work.

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