

Genetic Analysis of Molecular Mechanisms of Drug Resistance in *Mycobacterium tuberculosis* Against Four Major First-Line Anti-Tuberculosis Drugs (Isoniazid, Rifampin, Ethambutol, and Pyrazinamide)

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Abstract: Tuberculosis (TB) is a highly contagious and devastating disease that claims millions of lives annually. According to the World Health Organization (WHO), approximately 10.8 million people worldwide will be affected by TB in 2023, highlighting that TB remains the deadliest infectious disease globally. It is the second leading cause of death due to infectious disease. Additionally, the emergence of drug-resistant strains has created a significant challenge for the treatment of this disease. Approximately 25% of TB-related deaths are attributed to antimicrobial drug resistance. Various mechanisms contribute to the development of drug resistance in *Mycobacterium tuberculosis*; however, this resistance is primarily due to mutations in the target genes of antibiotics, which reduce the efficacy of anti-TB drugs. This study aimed to provide up-to-date and valuable information on the genetic mechanisms of *M. tuberculosis* resistance to major first-line anti-TB drugs. Understanding these mechanisms can open new avenues for researchers to treat TB and to overcome drug resistance.

Keywords: genetic analysis, drug resistance, *Mycobacterium tuberculosis*, first-line anti-TB drugs

Introduction

The *Mycobacterium* (*M.*) genus includes two significant human pathogens: *M. tuberculosis*, which is primarily responsible for tuberculosis (TB), and *M. leprae*, the bacterium that causes leprosy.¹ In 1993, the World Health Organization (WHO) declared TB a global health emergency, and it continues to be one of the most serious public health concerns worldwide.² In 2021, TB was reported to be the second leading cause of death due to infectious diseases.³ Every year, more than 2 million people lose their lives from TB, and an estimated one to two billion individuals worldwide are latently infected with the disease.⁴ According to the WHO Global Tuberculosis Report 2024, approximately 10.8 million people were diagnosed with TB worldwide in 2023, marking an upward trend compared to previous years. The report also indicates that TB has once again become the deadliest infectious disease globally, causing 1.25 million deaths in 2023, nearly twice the number of deaths attributed to HIV/AIDS.⁵ Along with increasing fatalities due to the synergistic effect of TB and HIV, the emergence of drug-resistant TB strains poses a significant challenge in disease management. The standard treatment for TB relies on a combination of first- and second-line drugs; however, the rise and spread of drug resistance, particularly in multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), represents a serious threat to disease control.⁶ It is estimated that nearly 25% of TB-related deaths are due to antimicrobial drug resistance.⁷ In 2021, approximately 450,000 cases of MDR-TB were reported, reflecting a 3.1% increase compared

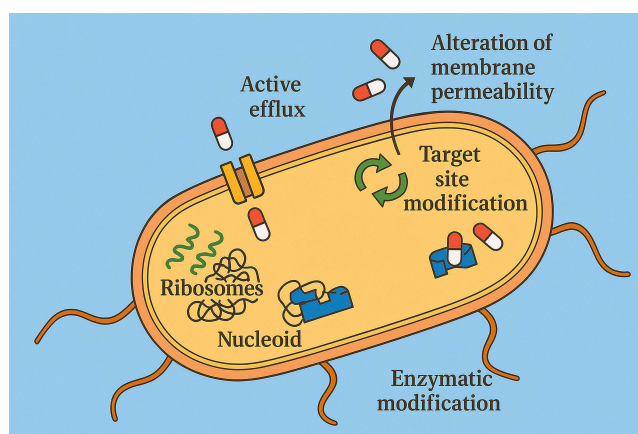


Figure 1 The primary mechanisms by which bacterial cells develop multidrug resistance: Alteration of membrane permeability to prevent antibiotics from entering the cell. Modification or alteration of antibiotic targets, rendering them ineffective. Enzymatic modification of antibiotics, leading to their inactivation. Expression of efflux pumps (EPs) that actively expel antibiotics from the cell.

with the previous year (2020).⁸ Additionally, in 2019, approximately 464,000 cases of rifampicin-resistant TB were documented globally, of which 78% were classified as MDR-TB.⁹

Drug resistance in *M. tuberculosis* primarily results from mutations in antibiotic target genes, leading to a reduction or complete loss of anti-TB drug efficacy. Furthermore, other mechanisms, such as changes in membrane permeability, modification or alteration of antibiotic targets, enzymatic modification of antibiotics, and expression of efflux pumps (EPs) that expel antibiotics from the bacterial cell, also contribute to resistance.¹⁰ Figure 1 illustrates the main mechanisms underlying bacterial resistance to antibiotics. Understanding these mechanisms is crucial for designing new therapeutic strategies and developing effective diagnostic methods.

The treatment duration for drug-sensitive TB is six months, whereas drug-resistant TB requires a prolonged treatment period of 18 to 24 months. These challenges create significant burdens for both patients and the strained public health systems. In many cases, patients discontinue treatment prematurely, leading to treatment failure and the emergence of *M. tuberculosis* strains with higher levels of drug resistance.¹¹

This article provides a molecular genetic perspective on the mechanisms of TB bacillus resistance to first-line drugs, including isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB), offering up-to-date and valuable insights into the resistance mechanisms against first-line anti-TB drugs.

Types of Drug Resistance in *M. tuberculosis*

Mono-Resistant TB

Refers to *M. tuberculosis* strains resistant to only one first-line drug, such as INH or RIF. This type of resistance can result from genetic mutations or efflux pump activity, and if not properly managed, may progress to more severe forms of drug resistance.¹²

Isoniazid-Resistant TB (Hr-TB)

Refers to *M. tuberculosis* strains that remain sensitive to rifampin, but resistant to isoniazid. Hr-TB is the most common form of drug-resistant TB worldwide.¹³

Rifampin-Resistant TB (RR-TB)

M. tuberculosis strains resistant only to RIF, although they may remain susceptible to other first-line drugs. This type of resistance is often an early indicator of potential MDR-TB and requires further investigation.¹⁴

Multidrug-Resistant TB (MDR-TB)

M. tuberculosis strains are resistant to at least two key first-line drugs, INH and RIF. MDR-TB requires treatment with second-line drugs, which are typically more toxic, more expensive, and less effective.¹⁵

Pre-Extensively Drug-Resistant TB (Pre-XDR-TB)

A recognized subtype of MDR-TB involves strains that, in addition to resistance to INH and RIF, also exhibit resistance to at least one fluoroquinolone while remaining susceptible to second-line injectable drugs. This condition is considered a potential precursor to XDR-TB.^{16,17}

Extensively Drug-Resistant TB (XDR-TB)

In addition to resistance to INH and RIF, MDR-TB strains also develop resistance to at least one fluoroquinolone and at least one second-line injectable drug (kanamycin, amikacin, or capreomycin). XDR-TB significantly complicates treatment and drastically lowers treatment success rates.¹⁸

Extremely Drug-Resistant TB (XXDR-TB)

M. tuberculosis strains were resistant to all first-line anti-TB drugs (INH, RIF, EMB, streptomycin (STR), PZA, and all second-line drugs (ofloxacin (OFX), ciprofloxacin (CIP), cycloserine (CYC), prothionamide (PTH), amikacin (AMK), kanamycin (KAN), ethionamide (ETO), para-aminosalicylic acid (PAS), and capreomycin (CAP)).^{18,19}

Totally Drug-Resistant TB (TDR-TB)

A newly identified category of *M. tuberculosis* strains has been found in Iran, Italy, South Africa, Russia, and India. These strains are resistant to all the currently available TB drugs. Although the WHO has not yet provided a formal definition for TDR-TB, it presents a serious public health challenge, as there are currently no effective treatment options. TDR-TB is the most severe form of drug resistance.^{19,20}

These classifications highlight the importance of monitoring TB drug resistance and emphasize the urgent need for the development of new treatment strategies and rapid diagnostic methods.

Mechanisms of Drug Resistance

Bacteria employ several molecular mechanisms to achieve antimicrobial resistance (AMR), including:

1. Barrier mechanisms: reducing cell wall permeability or actively expelling drugs via EPs.
2. Enzymatic degradation or inactivation: Breaking down or neutralizing the drug.
3. Modification of drug activation or metabolism pathways.
4. Drug target modification or amplification: Altering the target structure of the drug or increasing its expression.^{11,21}

M. tuberculosis uses these mechanisms to develop resistance. However, this study focused solely on drug resistance that arises from genetic mutations. For clearer and more intuitive presentation, these data are illustrated schematically in Figure 2.

First-Line Anti-Tuberculosis Drugs

Isoniazid (INH)

INH is a key first-line drug and a crucial component of TB treatment owing to its rapid bactericidal activity. It has been an essential component of TB therapy for decades. INH plays a significant role in reducing the bacterial load in sputum when combined with other anti-TB drugs in sensitive drug regimens. During the first two weeks of treatment, the bacterial count decreased by 10- to 100-fold (1–2 log). INH is widely used in the treatment of latent TB infection (LTBI).²² Despite its simple chemical structure comprising a pyridine ring and hydrazide group, its mechanism of action in the body and its impact on *M. tuberculosis* are highly complex and involve multiple processes. The major mechanisms include inhibition of cell wall synthesis, depletion of nucleic acid precursors, and metabolic suppression via nitric oxide (NO) production, all of which will be further explained.^{11,23}

Mechanism of Action

For decades, after INH was introduced as a first-line anti-TB drug, its exact mechanism of action has remained unclear. Recent studies have shown that the mycobacterial enzyme KatG activates INH through a peroxidative reaction, producing

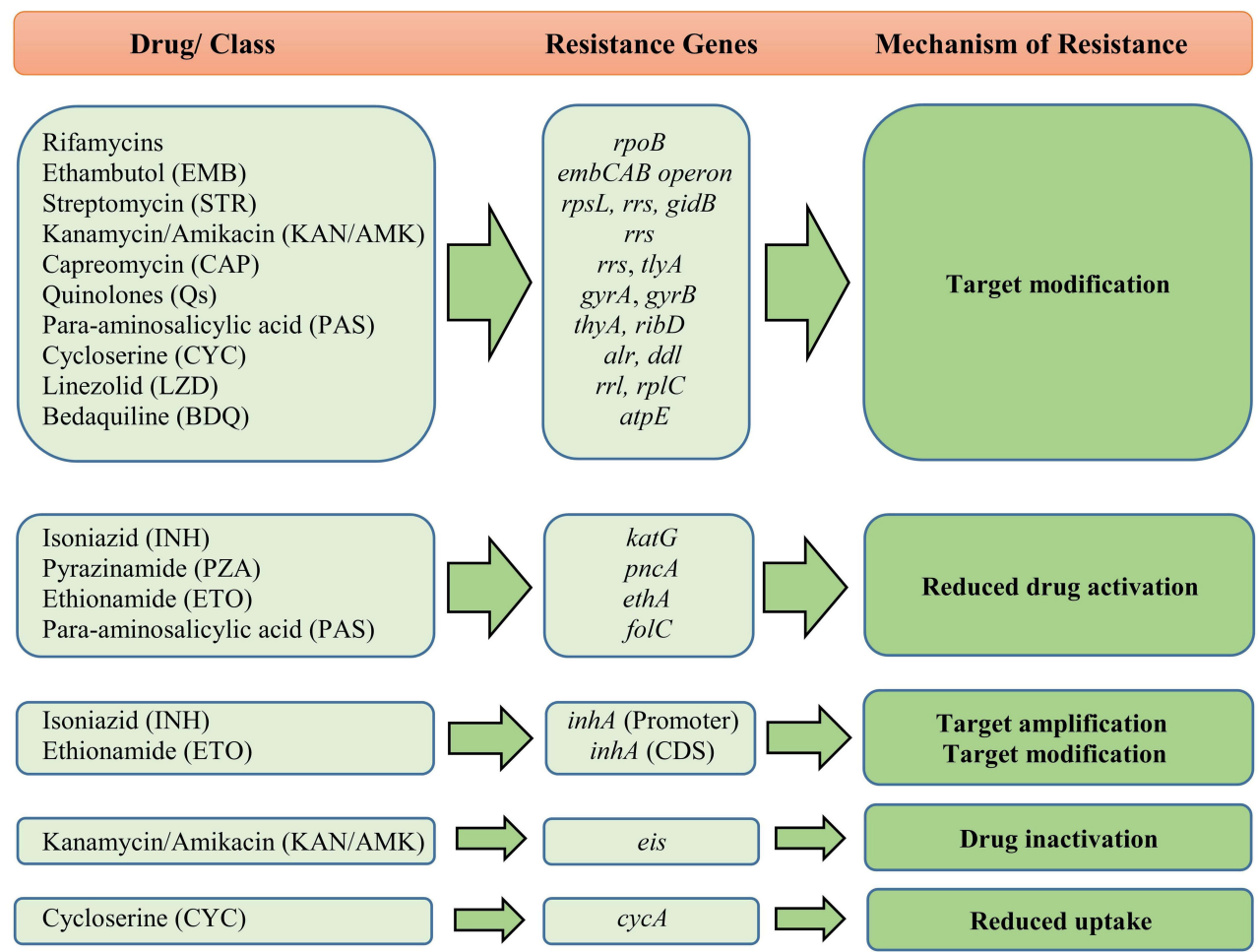


Figure 2 Schematic Representation of Drugs Commonly Used for TB Treatment, Associated Resistance Genes, and Mechanisms.

reactive species that combine NAD^+ and NADP^+ . These complexes strongly inhibit the key enzymes involved in lipid and nucleic acid synthesis. Additionally, some reactive species derived from INH, such as nitric oxide (NO), directly inhibit mycobacterial metabolic enzymes. The combination of these effects, including the inhibition of cell wall lipid synthesis, depletion of nucleic acid precursors, and metabolic suppression, contributes to INH's high potency and specificity.⁴ To fully understand INH's mechanism of action and resistance to INH, findings from multiple scientific fields, including molecular genetics, biochemical assays, and free-radical chemistry, must be integrated. This section explores these aspects in detail.

INH remains one of the most important anti-TB drugs and has maintained its primary role in TB treatment for more than 50 years since its discovery. However, its exact mechanisms of action are still a topic of debate. Here, we examined the different functional mechanisms of INH, which may act synergistically to explain its exceptional potency against *M. tuberculosis*.⁴

INH as a Prodrug Activated by KatG

INH is a prodrug activated by the mycobacterial enzyme KatG. It enters *M. tuberculosis* cells via passive diffusion but remains nontoxic until it is activated. The *katG* gene (Rv1908c/MT1959) encodes the KatG enzyme responsible for this activation. Once activated, INH binds to NAD, forming the INH-NAD complex, which subsequently inhibits the InhA protein. The *inhA* gene (Rv1484/MT1531) encodes InhA, an NADH-dependent enoyl-acyl carrier protein (ACP) reductase that is involved in fatty acid synthesis. This enzyme is part of the type II fatty acid synthase system (FAS-2), which plays a crucial role in mycolic acid biosynthesis. By inhibiting InhA, INH leads to the accumulation of long-chain fatty acids, a reduction in mycolic acid synthesis, and ultimately, cell death.¹¹

KatG is a multifunctional catalase-peroxidase enzyme. In addition to activating INH, it also exhibits NADH oxidase activity and contributes to the detoxification of reactive nitrogen species such as peroxynitrite.²⁴ In the absence of INH, KatG protects against oxidative stress in *M. tuberculosis*. However, during INH treatment, resistance mutations arise in *katG*, which reduce INH activation. These mutations include selective point mutations that alter the enzymatic function of KatG and reduce INH toxicity, while preserving some of the protective roles of KatG. One well-documented mutation, S315T, allows the enzyme to retain certain protective functions, but significantly diminishes its ability to activate INH.^{25,26}

Mechanism of INH Activation by KatG

KatG activates INH through peroxidation, which generates reactive species that cause intracellular damage. Various oxidizing agents, including superoxide, hydrogen peroxide, and simple alkyl hydroperoxides, facilitate the activation of INH by KatG. Even in the absence of these oxidants, INH and NADH undergo spontaneous oxidation under laboratory conditions, thus enabling KatG-mediated activation. Studies have shown that the oxidation of INH by KatG produces multiple reactive free radicals containing carbon, oxygen, and nitrogen. The most significant radicals include acyl, acylperoxo, pyridyl, and nitric oxide (NO). NO radicals derived from INH inhibit mycobacterial respiratory enzymes and contribute to bacterial eradication.^{27–29}

Direct Effects of INH-Derived Radicals

Unlike many other bacteria, *M. tuberculosis* lacks the *oxyR* gene, making it highly susceptible to oxidative stress and INH-induced damage. In the presence of INH, KatG is inhibited by its protective function and promotes the production of toxic radical species. These oxygen- and carbon-centered radicals can directly damage critical cellular components, including lipids, proteins, and nucleic acids.²⁸

Mechanisms of INH Resistance

There are two primary molecular mechanisms of INH resistance:

1. Loss of INH activation due to *katG* mutations
2. Increased expression of *inhA* or mutations that alter InhA's structure

Between 75% and 90% of INH resistance cases are associated with mutations in *katG* or the promoter region of *inhA*. Most INH-resistant clinical isolates carry mutations in *katG*, with various missense, nonsense, insertion, deletion, truncation, and complete gene deletion mutations being reported worldwide. Mutations in *katG* impair formation of the INH-NAD complex, leading to high resistance. Among these, the S315T mutation is the most frequently observed in clinical isolates. *KatG* polymorphisms can reduce or eliminate catalase and peroxidase activities, which are crucial for bacterial defense against reactive oxygen species (ROS) and virulence. However, unlike other *katG* mutations, the S315T mutation allows the bacterium to retain some peroxidase and catalase functions, thereby reducing the survival costs associated with drug resistance. This is likely the reason why S315T is widespread among drug-resistant *M. tuberculosis* isolates.^{23,30,31}

Although most clinical isolates resistant to INH have mutations in *katG*, some resistant isolates still possess the wild-type version of this gene, indicating that other resistance mechanisms are involved. These include changes in *inhA* expression, leading to structural alterations in the InhA target protein. It is well established that such changes are related to INH resistance. Studies have shown that Polymorphisms in the promoter region of *inhA*, particularly mutations at positions –15T and –8A, play a crucial role in conferring resistance to INH in *M. tuberculosis*.³² These mutations increase the expression of *inhA*, resulting in the overproduction of enoyl-ACP reductase, the primary target of INH, thereby reducing the effectiveness.^{30,33,34}

inhA Mutations and Cross-Resistance to Ethionamide

Under normal conditions, INH inhibits InhA, thereby blocking the synthesis of the essential fatty acids required for cell wall formation. However, promoter mutations at –15T and –8A increase *inhA* expression, leading to excessive production of InhA and reduced INH effectiveness. These mutations not only confer low-level INH resistance but also induce

cross-resistance to ethionamide (ETO), another anti-tuberculosis drug targeting InhA. The prevalence of these mutations varies by region but generally accounts for less than 20% of INH-resistant cases.^{35,36}

Although structural mutations in InhA are less common than those in the *inhA* promoter, one notable exception is S94A (serine-to-alanine substitution at position 94). This mutation reduces the binding affinity of InhA for NADH, resulting in low INH resistance.^{37–39}

Additional Genetic Factors in INH Resistance

Approximately 10% of INH resistance cases cannot be fully explained by *katG* or *inhA* mutations alone. Other genes, including *kasA*, *ahpC*, *ndh*, and the *ahpC-oxvR* intergenic region, have also been implicated in INH resistance, although their precise roles in clinical isolates remain unclear.^{37–39} Further research is needed to fully understand the contribution of these genes to INH resistance in *M. tuberculosis* strains.

Rifamycins

Rifamycins, including RIF, rifabutin (RFB), and rifapentin (RPT), play crucial roles in the sterilization of *M. tuberculosis* in infected patients. These antibiotics effectively target intracellular and dormant bacteria during different growth phases, making them essential for modern short-course TB treatment. Rifamycins have revolutionized TB therapy by shortening treatment duration and minimizing the risk of relapse. Although they differ in pharmacokinetics and pharmacodynamics in humans, their mechanisms of action and resistance patterns against mycobacteria are similar.⁴⁰

The primary target of rifamycins is the β -subunit of RNA polymerase (RNAP), which is encoded by the *rpoB* gene (Rv0667 in *M. tuberculosis* H37Rv and MT0695 in *M. tuberculosis* CDC1551). Rifamycins inhibit RNAP activity by binding to the RpoB protein and effectively blocking bacterial transcription. Because some levels of transcription continue even in dormant bacterial cells, rifamycins remain active against non-replicating mycobacteria, which underlies their sterilization effect. This property has significantly contributed to the shortening of the duration of TB treatment. Introduction of RIF alone reduced TB therapy from 18 to 9 months.⁴¹

Mechanism of Action

It has long been established that RIF exerts inhibitory effects after RNAP binds to DNA and initiates its transcription. Unlike what one might assume, RIF does not prevent the initiation of transcription but instead inhibits its elongation. Specifically, once the RNA transcript reaches 2–3 nucleotides in length, RIF blocks further synthesis. In 1999, Darst et al determined the first crystal structure of RNAP from *Thermus aquaticus*, followed by that of RIF-bound RNAP in 2001. These studies revealed that RIF binds deeply within the DNA/RNA channel of RNAP, but remains over 12 Å away from its active site.^{11,42}

Recent crystallographic studies on RNAP in *Escherichia coli* have revealed a more intricate transcription process comprising multiple steps:

1. Promoter binding: RNAP binds to the promoter region, forming a closed RNAP-promoter complex, in which the double-stranded DNA remains intact and does not enter the active site.
2. DNA unwinding: RNAP loads DNA and unwinds it within the active site, creating an RNAP-promoter open complex (RPoc).
3. Initial RNA synthesis: RNAP begins transcribing the first 10 nucleotides using a “scrunching” mechanism in which it remains fixed on the promoter while pulling downstream DNA into its cleft. This stage is known as the RNAP-promoter initial transcribing complex (RPitc).
4. Transcription elongation: RNAP escapes the promoter and continues RNA synthesis using a “stepping” mechanism, moving along the DNA template.^{43,44}

RIF specifically blocks step 3 by preventing RPitc formation, thereby halting transcriptional elongation. Studies have suggested that rifamycins inhibit transcription through a steric occlusion mechanism, physically obstructing the RNA exit path within the RNAP. RIF binds to the active center of the enzyme, preventing RNA chain extension beyond 2–3 nucleotides. Despite being positioned ~12 Å away from RNAP’s catalytic site of RNAP, where complementary

ribonucleotides are added to the growing RNA chain, the RIF-binding pocket within the β -subunit lies in the main DNA/RNA channel. By occupying this site, RIF effectively blocks the extension of nascent RNA molecules.^{43,44}

Mechanism of Resistance: *rpoB* Target Modification

In *M. tuberculosis*, similar to most bacterial species, RNA polymerase (RNAP) consists of four polypeptides, α , β , β' , and σ , forming a five-subunit enzyme organized as $\alpha_2\beta\beta'\sigma$. The individual genes *rpoA*, *rpoB*, and *rpoC* encode the α , β , and β' subunits, respectively, whereas 13 sigma factors can act as promoter-recognizing subunits.⁴⁵ The mechanism by which these mutations lead to RIF resistance has been inferred through X-ray crystallography studies of RNAP in other bacterial species, particularly *Thermus aquaticus* and *E. coli*.⁴⁶ More than 95% of RIF-resistant *M. tuberculosis* strains have non-synonymous mutations in *rpoB*, which encodes the β -subunit of RNAP. These mutations are concentrated within an 81-base-pair region of *rpoB*, known as the RIF resistance-determining region (RRDR).⁴⁷ Multiple mutations have been identified in various prokaryotic species within RRDR. However, the most common mutations—corresponding to codons in *E. coli* RNAP—include H526Y, H526P, H526D, H526R, N518 deletion, D516Y, D516V, G498E, G498Q, and S531L.^{11,48}

Fitness of RIF-Resistant Mutations and Compensatory Mechanisms

RIF inhibits RNAP function by binding to its β -subunit. Mutations in RRDR prevent the proper binding of RIF to RNAP while still allowing the enzyme to function. Bacterial strains with classic RRDR mutations are naturally selected when *M. tuberculosis* grows in RIF-containing environments. Laboratory-generated *M. tuberculosis* strains with *rpoB* mutations often exhibit reduced growth and survival rates. However, some clinical isolates of *M. tuberculosis* that carry the same RRDR mutations show less impairment in growth and survival than the wild-type strains. This suggests that these bacteria may have compensatory mechanisms that mitigate the negative effects of primary RIF resistance mutations, helping them to maintain their ability to grow. These compensatory mechanisms include secondary mutations in *rpoA* and *rpoC*, which counteract the effects of primary *rpoB* mutations and reduce the fitness cost of RIF resistance.¹¹

Ethambutol (EMB)

EMB, a first-line drug used for tuberculosis treatment, is commonly administered alongside RIF, INH, and PZA. It is a bacteriostatic agent that inhibits the biosynthesis of arabinogalactan in the bacterial cell wall, thereby preventing the replication of *M. tuberculosis*.⁴⁹ However, the precise underlying molecular mechanisms remain unclear.⁵⁰ Researchers have hypothesized that EMB has a synergistic effect with INH, which may be mediated by a transcriptional repressor that acts on the *inhA* gene. The *inhA* gene encodes enoyl-acyl carrier protein (CAP) reductase, an enzyme that is essential for maintaining the integrity of the bacterial cell wall. A study suggests that EMB binds to a TetR-family transcriptional regulator, increasing the sensitivity of the *inhA* gene to INH. Consequently, the bactericidal (bacteria-killing) effect of INH was enhanced.⁵¹

Mechanism of Action

The cell wall is a key drug target in *M. tuberculosis*. Arabinosyl transferases EmbA, EmbB, and EmbC play crucial roles in the synthesis of the mycobacterial cell wall complex and are considered targets of the anti-tuberculosis drug EMB. Mutations in these proteins may lead to the development of clinically resistant strains.⁵² These membrane-associated enzymes are involved in the polymerization of arabinan, a component of arabinogalactan and a major polysaccharide in the mycobacterial cell wall. Disruption of arabinan biosynthesis causes the accumulation of mycolic acids inside the cell, ultimately leading to cell death.⁵³ The *embCAB* operon, which consists of three genes encoding arabinosyl transferases *embC*, *embA*, and *embB*, has been identified in *M. tuberculosis*.⁵⁴ Using cryo-electron microscopy, Zhang et al identified the structure of heterodimeric EmbA-EmbB and homodimeric EmbC complexes. Their study revealed how donor and acceptor substrates bind to the active site, providing deeper insights into the arabinose transfer mechanism. EMB binds to the same site where the substrates attach to the EmbB and EmbC subunits, thereby inhibiting the enzyme through competitive binding. Mutations associated with EMB resistance are located near the drug-binding site.⁵⁵

Mechanism of Resistance: Target Modification in the *embCAB* Operon

Resistance to EMB is often associated with mutations in the *embCAB* operon (Rv3793-5 and MT3900-2). Specifically, mutations in *embC* and *embB* have been associated with drug resistance, with codons 306, 406, and 497 being the most frequently mutated sites.⁵⁴ In some studies, 50–70% of EMB-resistant clinical isolates showed polymorphisms at codon 306 of *embB*. However, this polymorphism has also been detected in EMB-susceptible isolates. Previously, this was attributed to limitations in phenotypic drug susceptibility testing, as *embB* mutations confer only low-level resistance to EMB.⁵⁶ Further evidence supporting the importance of codon 306 in EMB resistance comes from the finding that variations in the amino acid sequences of *M. leprae*, *M. abscessus*, and *M. chelonae* likely contribute to their natural resistance to EMB.⁵⁷

Because a significant percentage of EMB-resistant isolates lack mutations in *embCAB* genes, other unknown resistance mechanisms are likely involved. One potential mechanism involves *embR*, which regulates arabinosyl transferase activity under laboratory conditions. However, its role in clinical resistance remains unclear.⁵⁸ Recent genomic analyses of laboratory-selected EMB-resistant isolates have identified new genetic loci involved in resistance. For example, mutations in *Rv3806c*, which encodes the UbiA enzyme, have been detected in clinical EMB-resistant isolates. This enzyme is essential for the production of the precursors required by arabinosyl transferases. The combination of *ubiA* and *embCAB* mutations leads to increased EMB resistance levels.⁵⁹

Pyrazinamide (PZA)

PZA is a nicotinamide analog and a key first-line drug for TB treatment. Before the 1970s, PZA was mainly considered a second-line drug for the treatment of drug-resistant tuberculosis or relapse. However, studies have revealed that when combined with INH, PZA exhibits strong sterilizing activity when combined with INH. Subsequent clinical trials demonstrated a synergistic effect between PZA and RIF, showing that the treatment duration could be shortened from 12 months or more to 9 months with either RIF or PZA and to 6 months when both were used together. Since then, PZA has been included in the first-line tuberculosis treatment regimens in combination with RIF, INH, and EMB, forming the most effective treatment strategy for drug-sensitive tuberculosis. PZA also plays a critical role in MDR-TB treatment and is being evaluated in clinical trials, along with new anti-tuberculosis drugs.⁶⁰ Unlike many tuberculosis drugs, PZA has no effect on *M. tuberculosis* under standard laboratory conditions but becomes active in acidic environments (pH 5.5). Owing to this unique property, phenotypic drug susceptibility testing for PZA remains challenging, and no standardized method has been universally adopted. Consequently, many clinical laboratories do not routinely perform PZA susceptibility testing. Interestingly, while *M. tuberculosis* is susceptible to PZA, *M. bovis* and *M. bovis* BCG strains are naturally resistant to it.¹¹

The Role of PZA in Shortening Tuberculosis Treatment Duration

PZA plays a crucial role in reducing the duration of TB treatment from 9 to 12 months to 6 months. It is combined with INH and RIF and has demonstrated strong sterilizing activity in studies. PZA is used during the first two months of treatment, as prolonged use does not provide additional benefits.⁶¹ Recent efforts to optimize drug combinations with new TB medications in mouse models have shown that PZA is the only drug that cannot be omitted without compromising treatment efficacy, highlighting its unique importance.^{62–64}

Mechanism of Action of PZA

PZA is an unconventional and enigmatic drug that has puzzled researchers since its introduction in 1952. Unlike typical antibiotics that target actively growing bacteria, PZA is primarily effective against semi-dormant or non-replicating mycobacteria with low metabolic activity.⁶⁵ PZA is a prodrug that enters the bacterial cells in its inactive form. It is then converted into its active form, pyrazinoic acid (POA), by the enzyme pyrazinamidase (PZase), encoded by the *pncA* gene. In *M. tuberculosis*, this conversion occurs under acidic conditions. At a low pH, a portion of the POA becomes protonated, allowing it to easily penetrate the bacterial membrane. As the bacterial efflux system for POA is inefficient, acid accumulates in the cytoplasm, lowering the intracellular pH to unfavorable levels. This likely leads to the inactivation of essential enzymes such as fatty acid synthase I (FAS-I), which is crucial for synthesizing the vital lipids of bacteria. Ultimately, these metabolic disruptions result in the death of non-replicating *M. tuberculosis* cells. In contrast, at neutral pH, most POA remains in its anionic form and cannot enter the cell, which explains why PZA is only active under acidic conditions.⁵⁵

Mechanisms of Resistance to PZA

In 1967, McDermott et al demonstrated that resistance to PZA in *M. tuberculosis* is linked to the loss of nicotinamidase and pyrazinamidase (PZase) activity.⁶⁶ However, the exact resistance mechanism remained unknown until 1996, when researchers identified that mutations in the *pncA* gene, which encodes the PZase enzyme, are responsible for PZA resistance.⁶⁷

pncA Gene Mutations

Studies have shown that mutations conferring resistance to PZA occur at a relatively high frequency ($\sim 10^{-5}$). PZA-resistant strains typically lose PZase activity, and a strong correlation has been observed between PZase inactivation and PZA resistance in *M. tuberculosis*.⁶⁰ Mutations in *pncA*, which encodes pyrazinamidase/nicotinamidase (PZase), are the main mechanism of PZA resistance. These are mostly missense mutations leading to amino acid substitutions; however, some involve nucleotide insertions, deletions, or nonsense mutations within the structural region of *pncA* or its potential promoter region (such as position -11). These mutations are highly diverse and scattered throughout the gene. The extensive variability of *pncA* mutations, which are specific to PZA resistance, is not fully understood.^{68,69}

One study has investigated the effects of different *pncA* mutations on PncA enzymatic activity and PZA resistance. These findings indicated that reduced PncA enzymatic activity is generally associated with higher PZA resistance levels. However, this factor alone does not fully explain the wide range in resistance levels. Therefore, it was proposed that additional mechanisms contribute to the resistance to *pncA* mutations. Discrepancies between PZase activity and PZA resistance levels may also result from laboratory errors in PZA susceptibility testing. To test this hypothesis, researchers have suggested conducting studies using *M. tuberculosis* strains lacking PZase, but carrying mutated *pncA* variants with different levels of enzymatic activity.⁶⁰

Critical Regions in the PncA Structure

Despite the widespread distribution of *pncA* mutations, certain mutations are more frequently found in three specific regions: amino acids 3–17, 61–85, and 132–142. These regions contain catalytic and metal binding sites for PZase enzyme.⁷⁰ Structural studies of *M. tuberculosis* PZase have shown that mutations in some of these amino acids, such as C138, D8, K96, D49, H51, and H71, can alter the active site of the enzyme, leading to a loss of function. Other mutations, including those at F13, L19, H57 (observed as H57D in *M. bovis*), W68, G97, Y103, I113, A134, and H137, disrupt the active site structure, impair enzymatic activity, and confer PZA resistance.⁷¹

The crystal structure of PncA in *M. tuberculosis* has been determined, revealing significant differences in its substrate-binding pocket compared to PZases from *Pyrococcus horikoshii* and *Acinetobacter baumannii*. In *M. tuberculosis*, this region retains an Fe^{2+} ion coordinated to aspartate and three histidines, one of which is His57. In *M. bovis*, histidine is replaced by aspartate, rendering the bacterium inherently resistant to PZA. Investigating the structural impact of *pncA* mutations could provide further insight into the molecular basis of PZA resistance.⁷¹

PZA-Resistant Strains without *pncA* Mutations

Although > 99.9% of PZA-resistant strains harbor *pncA* mutations, rare cases of resistance without *pncA* mutations have been reported. Some of these cases may result from laboratory errors, but a small number of strains are genuinely resistant to PZA despite having an intact *pncA* gene.^{55,60}

These *pncA*-independent resistant strains fall into two categories:

1. High-level resistance with no PZase activity: These rare strains may result from mutations in an unknown regulatory gene that affects *pncA* expression.
2. Low-level resistance with retained PZase activity: These strains likely acquired resistance through alternative mechanisms, such as mutations in the *rpsA* gene (a proposed PZA target) or other unidentified genes. As these strains exhibited low-level resistance, they may still respond to PZA treatment.

Previous studies have shown that PZA resistance does not significantly affect the pathogenicity or transmissibility of these strains. Interestingly, some research suggests that PZA-resistant strains may possess enhanced virulence.^{55,60}

Mutations in the *rpsA* Gene

Recently, it was shown that some PZA-resistant isolates, such as DHM444, which does not have mutations in the *pncA* gene, and *M. canettii* have mutations in the drug target RpsA. This gene encodes ribosomal protein S1, which plays a key role in protein translation and is considered a drug target for PZA.⁷² Initially, it was thought that the C-terminal end of RpsA might be the drug-binding site; however, recent studies have shown that mutations in the middle regions or near the N-terminus of RpsA may also play a role in drug binding. For example, *M. canettii*, an organism from the *M. tuberculosis* complex that is naturally resistant to PZA and has no significant mutations in *pncA*, has several mutations in *rpsA*, including Thr5Ala, Pro9Pro, Thr210Ala, Glu457Glu, R474L, R474W, and E433D. Notably, target mutations in RpsA are generally associated with low PZA resistance. Further research is needed to evaluate whether these known mutations in *rpsA* are responsible for low-level resistance to PZA, further research is needed.³³

Mutations in the *panD* Genet

Although most cases of PZA resistance are caused by mutations in *pncA* and *rpsA*, some resistant strains lack these mutations. In a study that included 174 PZA-resistant mutations, 5 mutations in the *panD* gene were identified. This gene encodes aspartate decarboxylase, which plays a role in the synthesis of β -alanine (precursor of coenzyme A). PanD appears to be a potential target of PZA, and POA (the active form of PZA) may exert its antibacterial effect by inhibiting this pathway.⁷³

Host-Directed Activity of PZA

In 2009, Mendez et al demonstrated that PZA has an inhibitory effect on *Leishmania major* and increases the production of cytokines, such as IL-12, in macrophages. These results show that PZA has beneficial host-directed side effects that are effective in the treatment of leishmaniasis.⁷⁴ In similar studies on *M. tuberculosis*, treatment with PZA resulted in reduced secretion of pro-inflammatory cytokines and increased expression of anti-inflammatory genes, indicating the anti-inflammatory effect of PZA.⁷⁵ In experiments on PZA-resistant *M. bovis*, which is resistant to a mutation in *pncA*, treatment with PZA alone in BALB/c mice with a healthy immune system demonstrated a 4-log reduction in bacteria, whereas in nude mice, PZA had no additional lethality.⁷⁶ These results suggest that a healthy immune system is essential for the efficacy of PZA and bacterial killing, in addition to the need for activation by PncA.

Diagnostic Applications of PZA Resistance

As most PZA-resistant strains have mutations in the *pncA* gene, sequencing of this gene could be a rapid and effective diagnostic method for identifying resistance to PZA. Phenotypic susceptibility tests for PZA often yield false results, and sequencing of the *pncA* gene can help improve the treatment of patients with drug-resistant TB.⁶⁰

Diagnostic Tools for Drug-Resistant Tuberculosis

As discussed above, several genetic mutations in *M. tuberculosis* are commonly associated with bacterial resistance to first-line anti-tuberculosis drugs. Leveraging these genetic associations has enabled the development of molecular diagnostic tools, such as GeneXpert, Hain Line Probe Assay MTBDR*plus*, and MTBDR*sl*, which can accelerate the drug resistance identification process. The MTBDR*plus* test is designed to identify resistance to two major anti-tuberculosis drugs, RIF and INH, by detecting mutations in *rpoB* (for RIF resistance) and *katG* and *inhA* (for INH resistance). The MTBDR*sl* test is more advanced than MTBDR*plus*, and in addition to the genes and mutations present in MTBDR*plus*, it is designed to detect resistance to second-line anti-tuberculosis drugs (such as fluoroquinolones and amikacin).^{77,78}

GeneXpert is a PCR-based system that can be used in patient care centers to quickly and accurately analyze samples for the presence of the *M. tuberculosis* complex and to test for resistance to RIF. This system operates on the premise that nearly 95% of RIF-resistant cases result from mutations in *rpoB*. GeneXpert uses a molecular probe to detect the presence of a mutated *rpoB* gene as a marker for identifying drug-resistant TB.⁷⁹

Similarly, MTBDR*plus* uses specific probes to detect the *M. tuberculosis* complex by examining the presence of either wild-type or mutation-specific probes to identify mutations in the RRDR region of *rpoB* that cause resistance. This

test also examined mutations in *katG* and *inhA*, which lead to resistance to INH.⁷⁷ In addition, a similar version of this test is available for detecting mutations related to second-line drugs.

However, the genetic mutations used in these assays do not cover all known cases of drug resistance to the corresponding anti-tuberculosis drugs. This genomic ambiguity results in a relatively high rate of false-negative results in genetic-based tests, which is a major limitation to their clinical application. In fact, the MTBDR*plus* test is unable to identify 15–30% of INH-resistant cases and 5% of RIF-resistant cases. Improving the performance of these diagnostic tests requires identifying additional molecular mechanisms of resistance and including more genetic polymorphisms that can be used to detect a broader range of resistant phenotypes. Table 1 presents the diagnostic tools for drug-resistant tuberculosis (DR-TB), as outlined in the WHO Operational Handbook on Tuberculosis.⁸⁰

Whole-genome sequencing (WGS) is a promising and reliable method for rapid identification of drug resistance. A recent study showed that by conducting whole-genome sequencing of an initial isolate, it is possible to predict the presence of XDR-TB weeks before it is detected using culture-based methods.⁸¹

Table 1 Diagnostic Tools for Drug-Resistant Tuberculosis (DR-TB)

Tool/Method	Detected Drug Resistance	Genetic Targets	Turnaround Time	Sens/Spec	Advantages	Limitations	WHO 2024 Status
GenoType MTBDR <i>plus</i> v2.0	RIF/INH	<i>rpoB</i> (RIF), <i>katG</i> and <i>inhA</i> (INH)	~5 hours	RIF: 94–100%, INH: 92–96%	Rapid; detects both RIF and INH; direct sputum or culture use	Misses 15–30% of INH-resistant and ~5% of RIF-R due to rare or novel mutations	Endorsed as a moderate complexity NAAT
GenoType MTBDR <i>s</i> v2.0	FQs, AMK, KAN, CAP	<i>gyrA</i> , <i>gyrB</i> (FQ); <i>rrs</i> , <i>eis</i> (SLIDs)	~5 hours	FQ: ~93%, SLIDs: ~80%	Enables early MDR/XDR-TB detection; culture or smear-positive sputum	May miss resistance from rare/heterogeneous mutations (eg, in <i>gyrB</i> , <i>rrs</i> promoter)	Endorsed by WHO
Xpert MTB/RIF	RIF	<i>rpoB</i> (RRDR region)	~2 hours	Sens: ~89–94%, Spec: ~99%	Widely used, cartridge-based; minimal training needed	Does not detect INH or second-line drug resistance; false negatives from rare mutations	Endorsed as a simple NAAT
Xpert MTB/XDR	INH, FQs, ETO, SLIDs	<i>katG</i> , <i>inhA</i> , <i>gyrA</i> , <i>gyrB</i> , <i>eis</i> , <i>rrs</i>	~90 minutes	Varies (some gaps in mutation coverage reported)	Rapid, expanded resistance detection from single test	False negatives for rare mutations or heteroresistance; some limited field validation	Endorsed (2024) as advanced NAAT
tNGS (eg, Deeplex Myc-TB)	First- and second-line drugs: RIF, INH, FQ, AMK, BDQ, LZD, CFZ, DLM, etc.	Multiple genomic loci (~18+ genes)	2–5 days	High (>95% in validated panels)	Comprehensive, multiplexed; detects all WHO-prioritized resistance mutations	Requires lab infrastructure; sequencing platforms, data interpretation tools	Newly Endorsed (2024) for routine use
WGS	All known and novel drug resistance mutations, including new drugs	Entire <i>M. tuberculosis</i> genome (~4.4 Mbp)	~5–10 days	Very high (95–99% in validated pipelines)	Unparalleled resolution; detects rare/novel mutations; informs transmission studies	Requires high infrastructure, longer turnaround, not yet widespread in low-resource settings	Conditionally Recommended
Phenotypic DST	All drugs (gold standard reference)	Not genetic; growth-based (eg, MGIT)	1–8 weeks	Considered a reference; detects all resistance regardless of mutation	Can validate genotypic results	Slow; needs a high-level biosafety lab; not useful for immediate treatment decisions	Still used as a reference standard

Abbreviations: Sens, Sensitivity; Spec, Specificity; INH, Isoniazid; RIF, Rifampin; AMK, Amikacin; KAN, Kanamycin; BDQ, Bedaquiline; LZD, Linezolid; CFZ, Clofazimine; DLM, Delamanid; ETO, Ethionamide; TB, tuberculosis; FQs, Fluoroquinolones; RIF-R, Rifampin-resistant; MDR-TB, multidrug-resistant TB; XDR-TB, extensively drug-resistant TB; SLIDs, second-line injectable drugs; NAAT, nucleic acid amplification test; WGS, whole genome sequencing; tNGS, targeted next-generation sequencing; MGIT, mycobacterial growth indicator tube; DST, drug susceptibility testing; RRDR, RIF-resistance determining region.

Genomics and Drug Resistance

Despite significant advances in the identification of independent genes whose mutations lead to drug resistance, current models cannot explain some cases of resistance. One possible explanation is that multiple additive mutations combine to create phenotypic resistance. In recent years, several genomic studies have been conducted to identify resistance signatures that may be associated with individual resistance or multidrug-resistant phenotypes. Numerous recent WGS projects on resistant *M. tuberculosis* strains have led to the identification of more than 100 genetic regions associated with resistance, including those involved in the synthesis or regulation of surface lipids. As sequencing of *M. tuberculosis* strains becomes more common, these resistance signatures will be more precisely defined, enabling the identification of additional resistance mechanisms. Furthermore, these data can serve as the basis for algorithmic prediction tools to diagnose all types of drug-resistant TB.^{82,83}

Conclusion

Drug-resistant tuberculosis (DR-TB) remains one of the most formidable global health challenges, particularly in the face of rising MDR, XDR, and TDR strains of *M. tuberculosis*. This study has explored the molecular basis of resistance to four essential first-line anti-TB drugs — INH, RIF, EMB, and PZA— with a particular focus on the genetic mutations involved. By compiling the current understanding of resistance-associated genes such as *katG*, *inhA*, *rpoB*, *embB*, and *pncA*, we highlight the complex and multifactorial nature of TB drug resistance.

Understanding the genetic mechanisms of resistance is not only critical for developing new treatment strategies but also vital for enhancing molecular diagnostic tools. Despite advances in diagnostic technologies like GeneXpert, MTBDR*plus*, MTBDR*sl*, and WGS, gaps remain in detecting all resistance-conferring mutations, especially those beyond the commonly known targets. These limitations underscore the need for expanded mutation databases and improved algorithms for resistance prediction.

Moving forward, integrating molecular insights into clinical decision-making, tailoring therapy based on rapid genotypic testing, and investing in global surveillance of resistance mutations will be key strategies in the fight against TB. Continued research into lesser-known resistance mechanisms and host-pathogen interactions may also open new therapeutic avenues. Ultimately, a multifaceted approach combining genomics, diagnostics, and novel therapies will be essential to curb the TB epidemic and reduce the global burden of drug-resistant TB.

Clinical Trial Registration

This study does not involve a clinical trial and, therefore, does not require registration.

Data Sharing Statement

The data supporting the findings of this study are available upon reasonable request from the corresponding author. Owing to privacy and ethical considerations, raw patient data cannot be shared publicly.

Ethics Statement

This study was conducted in accordance with ethical guidelines and approved by the relevant institutional ethics committee. None of the human or animal subjects were included in the experimental procedures.

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