Drug Resistance Mechanisms in Tuberculosis

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The increased incidence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* (Mtb) strains, defined as resistant to at least isoniazid and rifampin, the two highly bactericidal first-line drugs, is a major concern for tuberculosis (TB) control. The worldwide estimate of almost half a million incident cases of MDR/rifampin-resistant TB, is causing increasing concern. In this view, it is important to continuously update the knowledge on the mechanisms involved in the development of drug-resistant TB. Clinical, biological and microbiological reasons account for the generation of resistance, including: (i) nonadherence of patients to their therapy, and/or errors of physicians in therapy management, (ii) complexity and poor vascularization of granulomatous lesions, which obstruct drug distribution to some sites, resulting in resistance development, (iii) intrinsic drug resistance of tubercle bacilli, (iv) formation of non-replicating, drug-tolerant bacilli inside the granulomas, (v) development of mutations in Mtb genes, which are the most important molecular mechanisms of resistance. Here, a piece of information on the interplay of these factors is provided, to facilitate the clinical and microbiological management of drug-resistant TB at the global level, with attention also to the most recent diagnostic methods.

Introduction

*Mycobacterium tuberculosis* (Mtb) is the etiologic agent of tuberculosis (TB), the leading cause of death from a single infectious disease agent worldwide [1]. In 2018, the World Health Organization (WHO) estimates of the global burden of TB were 10 million cases and 1.45 million deaths. The current antibiotic treatment of drug-susceptible TB requires administration of a combination therapy for 6 months, including the first-line drugs rifampin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) for 2 months, followed by RIF and INH for 4 months.

For a number of reasons, development of drug-resistant Mtb strains may occur, including multidrug-resistant (MDR: resistant at least to INH and RIF) and extensively-drug-resistant (XDR) strains [MDR resistant to a fluoroquinolone (FQ) and a second-line injectable drug [kanamycin (KM), amikacin (AM), capreomycin (CM)] [2]]. WHO reported that in 2018 there were an estimated 484,000 incident cases of MDR/rifampin-resistant (RR) TB cases, including about 378,000 MDR-TB cases and 214,000 deaths. The average proportion of MDR-TB cases with XDR-TB was 6.2% [1]. At the global level, 3.4% of new cases (patients never treated with anti-TB medicines, or treated for < 1 month) and 18% of previously treated cases (patients treated for ≥1 month in the past) had MDR/RR-TB, with the highest proportion occurring in the former Soviet Union (FSU) countries. In the low incidence countries of the European Economic Area, the MDR-TB was more prevalent among migrants (particularly from the FSU) than the native population [3][4][5][6][7][8].

Drug Resistance Mechanisms

If the 6-months combination therapy for the treatment of drug-susceptible TB is adequately taken, patients achieve cure rates of >95%. The resistance developed by Mtb to any antimicrobial agent is not due to a single mechanism, but to the interplay of biological, clinical and microbiological reasons, including

1. Nonadherence of patients to therapy and/or errors of physicians in the therapy management (human errors), that increases the risk of developing genetically drug-resistant bacilli [5][6];

2. Complexity and poor vascularization of granulomatous lesions, which obstruct drug distribution to some sites, leading to suboptimal drug concentration and development of phenotypic and genetic resistance [7][8];

3. Naturally occurring high levels of antibiotic resistance in tubercle bacilli (intrinsic resistance) [8][9][10][11];

4. Formation of non-replicating (NR) drug-tolerant bacilli inside the granulomas (phenotypic resistance) [9][11][12];

5. Development of genetically resistant bacilli by chromosomal mutations (acquired resistance) [4][13][14][15][16].
Human Errors

Human errors may contribute to development of drug-resistance. Two pathways lead to genetic resistance: (i) primary resistance, when a person is infected with a drug-resistant strain, and (ii) acquired resistance, when a person infected with a drug susceptible strain is inadequately treated with drugs, allowing the selection of resistant mutants \[17\]. The first case mostly occurs in highly crowded communities (e.g., prisons), or in countries with high MDR-TB prevalence, where it is essential to rapidly diagnose and treat patients, so as to reduce transmission \[8\]. In the second case, it is essential to follow the WHO recommendations on how to adequately treat the TB patients whose disease is caused by a drug-susceptible strain. The clinicians need also to ensure that infection control measures are established, particularly when MDR-patients are hospitalized.

WHO identified a number of factors contributing to poor treatment outcomes, including acquisition of acquired drug-resistant TB \[17\]. They were: (i) Inappropriate treatment by health care providers (inappropriate or absent guidelines, poor training of physicians and nurses, sub-optimal education of patients, poor management of adverse drug reactions, no monitoring of treatment, poorly organized or funded TB control programs); (ii) Inadequate drug supply (poor quality medicines, stock-outs, poor storage conditions, wrong dose or combination); (iii) Inadequate drug intake or treatment response by patients (lack of information on treatment adherence, adverse effects, malabsorption).

Common clinical errors in MDR-TB management, particularly in developing countries, include the addition of a single drug to a failing regimen, failure to recognize existing drug resistance, failure to provide directly observed therapy and to manage nonadherence, suboptimal dosages of second-line drugs to decrease side effects, drug treatment based on clinical facts while waiting for drug susceptibility testing (DST) results \[5,17\]. In any case, it is important to know that only drug combinations decrease the risk of selection of resistant strains.

Complexity of TB Granulomas

Long lasting therapies are also attributable to the complex pathology of TB. In the lungs of TB patients, a spectrum of heterogeneous granulomatous lesions coexist, ranging from well-vascularized cellular granulomas, in which a rim of lymphocytes surrounds macrophages and neutrophils, to avascular caseous granulomas, characterized by a necrotic center with a cheese-like aspect (caseum) formed by the lysis of host cells and bacteria \[18,19\]. In these lesions, tubercle bacilli range from actively replicating (AR) stages, particularly in cellular granulomas, to dormant, slowly-replicating or NR stages, typical of hypoxic caseous granulomas \[20,21\].

The current 4-drugs therapeutic regimen (RIF-INH-PZA-EMB) is effective against AR intracellular bacilli in cellular granulomas, while NR extracellular bacilli localized in pH-neutral, caseous granulomas are refractory to drug action \[8,12,22,23,24\]. The necrotic center of caseous granulomas contains NR bacilli phenotypically resistant to several drugs (drug-tolerant persisters), with the exception of rifamycins, which are known to sterilize caseum in ex-vivo assays \[24,25\]. Spatial and temporal differences in drug distribution and the kinetics of accumulation of drugs in specific lesion compartments may create local windows of monotherapy that increase the risk of the emergence of drug-resistance \[8,26\]. In this view, drug combinations should contain complementary drugs preferentially distributing in lesions in which their most vulnerable target population resides \[8\].

In the event of caseous granulomas expansion, the necrotic centers fuse with the airway structures of bronchi to form pulmonary cavities in which are found both extracellular bacilli in the liquefied caseum and intracellular bacilli derived from the lysis of infected macrophages of the cavity walls. In contact with the atmospheric oxygen, these bacilli rapidly proliferate in the lumen of cavities, and later appear in the sputum of TB patients \[3\]. Due to high bacterial load in pulmonary cavities, genetically resistant bacilli with chromosomal mutations may be generated, playing an important role in the development of resistance \[7\]. Drug-specific gradients in the walls of human pulmonary cavities were reported to be associated with the development of acquired resistance in patients with MDR-TB, due to the low level of some drugs in the cavities centers, where there is a high number of replicating bacilli \[27\].
Intrinsic Drug-Resistance

During the evolution, Mtb developed mechanisms of intrinsic resistance involving cell envelope, efflux systems and other mechanisms (drug degradation and modification, target modification). Some examples of these mechanisms are provided in the following sections.

Cell Envelope

The constituents of the mycobacterial cell envelope are: the cytoplasmic membrane, the periplasmic space (PS), a network of peptidoglycan (PG), the arabinogalactan (AG), the long-chain mycolic acids (MA) and the capsule, made of a loose matrix of glucans and secreted proteins [28]. As to the first-line TB drugs, the bactericidal agent INH inhibits MA synthesis, while the bacteriostatic EMB inhibits AG synthesis and may sensitize Mtb to other drugs [29].

It is assumed that the innermost hydrophilic layers of PG and AG hinder the penetration of hydrophobic molecules. Instead, in the external part of the envelope, the hydrophobic MA layer formed by long-chain fatty acids restricts the penetration of hydrophilic drugs [26]. In principle, more lipophilic drugs, such as rifamycins, macrolides, and some FQs, diffuse by passive transport into and through the lipid-rich cell wall [29,30]. However, the issue is perhaps more complex, since some studies showed that lipophilicity is an important but not exclusive factor of compound permeability [31,32].

Drug Efflux

Efflux pumps (EPs) are transmembrane proteins that provide resistance by expelling the drugs from the interior to the exterior of the cell. Five EP families are known, organized on the basis of energetic and structural characteristics: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) superfamily [9,11,20,23,24]. The ATP-energized ABC members are primary transporters, while the others are secondary transporters energized by proton gradients (MFS, SMR, RND) or sodium gradients (MATE). The EP of Mtb belongs to the ABC (representing 2.5% of the entire Mtb genome), MFS and RND superfamilies, and to the SMR family.

Following exposure of Mtb to sub-inhibitory concentration of INH and EMB, EP genes are overexpressed resulting in the development of low-level resistance for a prolonged period of time. After several weeks, a high level of acquired resistance develops, caused by chromosomal mutations in the genes encoding the target proteins [35,36]. These observations indicate that inappropriate TB treatment may generate pressure by sub-inhibitory drug concentrations that increase drug efflux, allowing a subsequent selection of mutants with high-level resistance [30,34].

Several EPs are known to be associated with resistance. For instance, Mtb exposure to INH induces overexpression of MmpL7 and mmr EP genes [37,38]. Furthermore, several EPs are involved in resistance to several drugs. Thus, the EP Tap mediates low-level resistance to tetracycline (TC) and aminoglycosides, whereas EPs encoded by the Rv0194 gene are associated with resistance to TC, β-lactams, streptomycin, chloramphenicol, and vancomycin. Mutations in the Rv0678 gene caused an up-regulation of the transport protein MmpL5, which caused EP-mediated cross-resistance to both bedaquiline (BDQ) and clofazimine (CFZ) [37,39,40]. This is a potentially dangerous evolution of Mtb against antibiotics particularly in recent times, since BDQ and CFZ have just been included in the new WHO treatment guidelines of MDR/XDR-TB [41,42].

Other Mechanisms

The most important mechanisms of the intrinsic drug resistance of Mtb are considered to be the lipid-rich cell wall and the EP, but other systems are known to neutralize toxic chemicals and antibiotics, including drug inactivation or modification, and target modification.

Among drug inactivating enzymes, Mtb β-lactamases, which are probably localized in the PS, are less effective than those of other bacteria to hydrolyse β-lactams, but their activity, together with slow penetration across the cell wall and low affinity for penicillin-binding proteins, is good enough to render Mtb intrinsically resistant to most β-lactams [9,11].
As to aminoglycosides (KM, AM) and cyclic peptides (CM), Mtb is able to inactivate them by acetylation performed by the enhanced intracellular survival protein encoded by eis, whose expression is upregulated by the MDR transcription regulator WhiB7 \[^{[11]}\]. Promoter mutations lead to an overexpression of eis, resulting in low-level resistance to KM, but not AM \[^{[43]}\].

*M. tuberculosis* naturally resistant to macrolides (e.g., clarithromycin and azithromycin) because of the inducible *erm(37)*, a ribosomal RNA methyltransferase which alters ribosomes by methylating the 23S rRNA \[^{[44,45]}\].

### Phenotypic Drug-Resistance

Caseous granulomas and the cavities of the lungs of TB patients harbor subpopulations of NR bacilli which are phenotypically drug-resistant but genetically susceptible, commonly referred to as persisters. Characterized by a transient, non-heritable drug tolerance, persisters are capable of withstanding bactericidal drug concentrations, and once the antibiotic is removed, to resume growth with genetic features identical to the original strain.

The level of resistance to different antimicrobial agents varies with the in vitro stress model used \[^{[46,47,48,49]}\], including hypoxia (Wayne dormancy model) \[^{[50,51]}\], nutrient starvation \[^{[52]}\], acids and/or nitric oxide \[^{[53,54]}\], stationary phase \[^{[55]}\], antibiotic-starved strains \[^{[56]}\] and others, or their variants. In the Wayne model, in which dormant bacilli are obtained by a gradual adaptation to anabiosis through the self-generated formation of an oxygen gradient, nonreplicating persistence (NRP) stages 1 and 2 were observed \[^{[50]}\]. NRP-2 cells developed a thickened outer layer that helped in restricting RIF entry \[^{[52]}\]. Our group used the Wayne model at different pHs: pH 6.6, the pH or culture media \[^{[58]}\], pH 5.8, to mimic the environment of cellular granulomas \[^{[59]}\], pH 7.3, to mimic the environment of caseous granulomas \[^{[60]}\]. We found that at pH 5.8, several drugs killed NR bacilli, with the best being the rifamycins RIF and rifapentine (RFP), while at pH 7.3, only RIF and RFP killed dormant bacilli out of 12 drugs tested \[^{[60]}\]. Since the rifamycins were the only agents sterilizing caseum obtained from rabbits \[^{[24,25]}\], our model could mimic caseum to measure drug activity against NR Mtb in this environment. In hypoxia at pH 7.3, we found that RIF plus nitazoxanide (a nitro-compound for anaerobic infections) killed NR Mtb cells, while the combination currently used for human TB therapy (RIF-INH-PZA-EMB) did not \[^{[61]}\]. Dormancy is not necessary or sufficient for Mtb persistence, indicating that persistence is a phenomenon more complex than dormancy, and that additional characteristics are needed to define the persister phenotypes, which depends on the NR model used \[^{[52]}\].

Two kinds of persisters are known \[^{[49]}\]: (i) Class I, rare, generated in a replicating population, formed continuously and in a purely stochastic manner. They are bacilli phenotypically tolerant to different antibiotics by different mechanisms, and it is likely that the overall population can be killed by drug combinations; (ii) Class II, abundant, involving almost all of the cells in a population, e.g., in the stationary phase, hypoxic conditions, nutrient starvation. Growth arrest is associated with resistance to a large number of drugs, and it is likely that new kinds of antibiotics are necessary to overcome these cells \[^{[46]}\].

Interestingly, RIF-resistant or moxifloxacin (MXF) resistant mutants carrying mutations in *rpoB* or *gyrA* genes emerged at high frequency from the persistent phase of Mtb cells exposed to RIF for prolonged periods. These cells carried elevated levels of the hydroxyl radical, which inflicted genome-wide mutations facilitating resistance to the same, or another, antibiotic \[^{[63,64,65]}\]. In consideration of the long TB therapy, these observations may have clinical significance in the emergence of drug-resistant mutants if local monotherapy occurs in patients who do not correctly take multi-drug TB therapy. In this view, it was postulated that persisters behave as an evolutionary reservoir from which drug-resistant mutants can emerge \[^{[19]}\].

### Acquired Drug-Resistance and drug susceptibility testing

A cocktail of different drugs is used to treat TB. Each molecule binds to one or more target, thus inhibiting their functions. The continuous drug exposure during lengthy treatments and the noncompliance of patients to drug regimens, pushes Mtb to select for mutations in genes encoding drug targets, responsible for development of the majority of resistances in...
clinical strains [11]. A list of the major target genes that, in the case of mutation, confer resistance to the drugs of the recently reported WHO treatment groups A, B and C [41,42], is shown in Table 1 [66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95]. Many excellent reviews report the genetic mechanisms involved in this resistance to RIF, INH, KM, CM and other drugs [96,97,98].

Phenotypic testing is still considered a gold standard for Mtb DST, which is accurate, but takes at least two weeks for results [67]. However, a pivotal role has been recently played by the more and more rapid molecular methods to diagnose drug-resistant TB by the identification of chromosomal mutations, including line probe assays, the Xpert MTB/RIF system (Cepheid, Sunnyvale, CA, USA), target gene sequencing, whole genome sequencing (WGS), point-of-care nucleic acid amplification devices [96,97,98].

The Treatment Action Group (TAG) recently released the pipeline report 2019 on TB diagnostics [69]. The TAG-stratified DST tests for decentralized and centralized laboratories. As to the decentralized tests, the Xpert MTB/RIF assay (sensitivity and specificity for RIF resistance of 96% and 98%, respectively) was recommended by the WHO in 2010, and entered in the market in the same year. The sensitivity of this assay increased with the 2017 rollout of the Xpert MTB/RIF Ultra cartridge. In 2020, there is expected the WHO evaluation and market entry of Xpert XDR, which will detect resistance to INH, MFX, AM, KM, ofloxacin [99]. In 2013, another company (Molbio Diagnostics, Goa, India) released its systems Truenat MTB and Truenat MTB-RIF Dx onto the Indian market [99]. In January 2020, a rapid WHO Communication reported that the Truenat systems MTB, MTB Plus and MTB RIF Dx assays showed comparable accuracy with Xpert MTB/RIF and Xpert Ultra for Mtb detection (Truenat MTB and Truenat MTB Plus), and for sequential RIF resistance detection (Truenat MTB RIF Dx) [100]. Furthermore, the data for Truenat MTB RIF Dx showed similar accuracy to the WHO approved commercial line probe assays indicated by the TAG for centralized DST [GenoType MTBDRplus Version 2.0 (Hain Lifescience, Nehren, Germany) and Nipro NTM+MDRTB detection kit2 (Nipro, Osaka, Japan)] [99,100]. Other systems marketed in 2015–2019 and on the pathway to the WHO evaluation for the centralized determination of molecular resistance to INH and RIF are: Cobas MTB-RIF/INH (Roche, Basel, Switzerland), BD MAX MDR-TB (Becton Dickinson, Sparks, MA, USA), real-time MTB-RIF/INH Resistance assay (Abbott, Abbott Park, IL, USA) and FluoroType MTBDR version 2.0 (RIF, INH) (Hain Lifescience) [99,101,102,103].

Finally, the WGS technology is capable of identifying the complete drug-resistance profile of an Mtb strain, ideally enabling clinicians to obtain the best anti-TB treatment [67,98,100,104]. However, more data are still needed to correlate genetic mutations with phenotypic resistance, in order to definitely guide the clinical care.

In this view, the initiative of the Comprehensive Resistance Prediction for Tuberculosis: an International Consortium (CRyPTIC) project aims at understanding the relationship between genotypes and resistance by sequencing 100,000 whole TB genomes from various countries, in parallel with comprehensive DST assays. Overall, at this stage, the WGS still needs more studies, but it is commonly believed that this technology will be the future of rapid, centralized DST [99,105].

Table 1. Drugs of the World Health Organization (WHO) groups A, B and C, and list of the most common drug resistance-related target genes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug*</th>
<th>Target Gene/s</th>
<th>Gene Product (Function Affected)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>LFX or MFX</td>
<td>gyrA</td>
<td>DNA gyrase, subunit A (DNA replication)</td>
<td>[66,67]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gyrB</td>
<td>DNA gyrase, subunit B (DNA replication)</td>
<td>[68,69]</td>
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<td></td>
<td></td>
<td>Description</td>
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<tr>
<td>BDQ</td>
<td>atpE</td>
<td>ATP synthase, subunit F0 (ATP synthesis)</td>
<td>[69][70]</td>
<td></td>
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<tr>
<td></td>
<td>rv0678</td>
<td>Transcriptional regulator (drug efflux)</td>
<td>[69][70]</td>
<td></td>
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<tr>
<td>LZD</td>
<td>rplC</td>
<td>50S ribosomal protein L3 (protein synthesis)</td>
<td>[69][71]</td>
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<tr>
<td></td>
<td>rrl</td>
<td>23S RNA (protein synthesis)</td>
<td>[69][71]</td>
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<tr>
<td>B</td>
<td>CFZ</td>
<td>rv0678 Transcriptional regulator (drug efflux)</td>
<td>[69][72]</td>
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<tr>
<td></td>
<td>rv1979c</td>
<td>(Possible permease involved in aminoacid transport)</td>
<td>[69][72]</td>
<td></td>
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<tr>
<td></td>
<td>rv2535c</td>
<td>(PepQ putative aminopeptidase)</td>
<td>[72][73]</td>
<td></td>
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<tr>
<td>CS or TRD</td>
<td>alr</td>
<td>Alanine racemase (peptidoglycan synthesis)</td>
<td>[74]</td>
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<tr>
<td>C</td>
<td>EMB</td>
<td>embCAB Arabinofuranosyltransferases (arabinogalactan synthesis)</td>
<td>[75][76]</td>
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<tr>
<td></td>
<td>ubiA</td>
<td>Phosphoribosyltransferase (cell wall synthesis)</td>
<td>[77][78]</td>
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<tr>
<td>DLM</td>
<td>ddn</td>
<td>Deazaflavin (F_{420})-dependent nitroreductase (mycolic acid synthesis)</td>
<td>[79]</td>
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<tr>
<td></td>
<td>fgd-1</td>
<td>Glucose-6-phosphate dehydrogenase (F_{420} synthesis)</td>
<td>[79]</td>
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<tr>
<td></td>
<td>fbiA</td>
<td>Protein FbiA (F_{420} synthesis)</td>
<td>[79]</td>
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<td></td>
<td>fbiB</td>
<td>Protein FbiB (F_{420} synthesis)</td>
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<td>Gene</td>
<td>Description</td>
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<tr>
<td><em>fbiC</em></td>
<td>Protein FbiC (F&lt;sub&gt;420&lt;/sub&gt; synthesis)</td>
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<tr>
<td><em>pncA</em></td>
<td>Pyrazinamidase (conversion of pro-drug PZA into pyrazinoic acid, resulting in dysfunctions of membrane potential)</td>
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<tr>
<td><em>rpsA</em></td>
<td>30S ribosomal protein S1 (m-RNA trans-translation)</td>
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<tr>
<td><em>panD</em></td>
<td>Aspartate decarboxylate (panthotenate synthesis)</td>
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<tr>
<td><em>clpc1</em></td>
<td>ATP-dependent ATP-ase (protein degradation)</td>
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<tr>
<td><em>rv2518c</em></td>
<td>LdtB, nonclassical, L,D-transpeptidase (peptidoglycan synthesis)</td>
<td></td>
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<tr>
<td><em>rv3682</em></td>
<td>PonA2, penicillin-binding protein (peptidoglycan synthesis)</td>
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<tr>
<td><em>Rv2068c</em></td>
<td>blaC (major b-lactamase)</td>
<td></td>
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<tr>
<td><em>rrs</em></td>
<td>16S ribosomal RNA (protein synthesis)</td>
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<td><em>rpsl</em></td>
<td>ribosomal protein S12 (protein synthesis)</td>
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<tr>
<td><em>rrs</em></td>
<td>16S ribosomal RNA (protein synthesis)</td>
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<tr>
<td><em>gidB</em></td>
<td>(putative 16S rRNA methyltransferase)</td>
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<tr>
<td><em>rv0565c</em></td>
<td>Monoxygenase (activation of prodrugs ETO and PTO)</td>
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</table>
ethA  - Monooxygenase (activation of pro-drugs ETO and PTO)  [10][91]

mymA  - Monooxygenase (activation of pro-drugs ETO and PTO)  [90][92]

katG  - Catalase-peroxidase (activation of pro-drugs ETO, PTO, INH)  [91]

inhA  - Enoyl-ACP reductase (mycolic acid synthesis)  [67][91]

PAS  - ThyA  Thymidylate synthase  [14][93]

folC  - Dihydropholate synthase  [14][94]

dfrA  - Dihydropholate reductase  [14][94]

(* LFX, levofloxacin; MFX, moxifloxacin; BDQ, bedaquiline; LZD, linezolid; CFZ, clofazimine; CS, cycloserine; TRD, terizidone; EMB, ethambutol; DLM, delamanid; PZA, pyrazinamide; IPM-CLN, imipenem-cilastatin; MPM, meropenem; AM, amikacin; SM, streptomycin; ETO, ethionamide; PTO, protionamide; PAS, para-aminosalicylic acid.

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