Chapter 7

The Physiology of *Mycobacterium tuberculosis* in the Context of Drug Resistance: A System Biology Perspective

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Additional information is available at the end of the chapter

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Abstract

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* (*Mtb*), is the main cause of death due to an infectious disease. After more than 100 years of the discovery of *Mtb*, clinicians still face difficulties finding an effective treatment for the increasing number of drug-resistant cases. The difficulties in the clinical setting can be related to the slow pace at which the understanding of the physiology of this bacterium has occurred. *Mtb* is distinct from other microorganisms not only due to its slow growth and difficulties to study in the laboratory, but also due to its inherent physiology such as its complex cell envelope and its metabolic pathways. Understanding the physiology of drug susceptible and resistant *Mtb* strains is crucial for the design of an effective chemotherapy against TB. This chapter will review the mycobacterial cell envelope and major physiological pathways together with recent discoveries in *Mtb* drug resistance through different "omics" disciplines.

Keywords: drug resistance, physiology, systems biology, proteomics, genomics, lipidomics

1. Introduction

The history of tuberculosis (TB), the disease caused by *Mycobacterium tuberculosis* (*Mtb*), has a remarkable involvement in human history; particularly in the evolution of human society and in the development of many scientific disciplines. TB has a negative role in many pages of human history, taking lives of many renowned artists, politicians, as well as poor, wealthy, young, or adult individuals. After its apparent "resurgence" at the end of the last century with the concomitant arises of HIV/AIDS cases, TB has been mainly associated with poverty and

immunosuppression. *Mtb* was the model microorganism that inspired Koch to develop his postulates which are a cornerstone of sciences such as Microbiology and Immunology. What Robert Koch never probably imagined was that over a century later, this disease was going to continue as the leading cause of death, mostly because of the increasing number of drug-resistant TB cases. Regarding TB treatment, it is discouraging to see how this lethal disease only started to be cured and controlled during 1950s with the discovery of the first chemotherapy. It is hard to imagine the labor of a clinician taking care of TB patients before 1950s without an available treatment option. However, this same scenario is similar to what many health providers face nowadays with the current spreading of drug-resistant cases, particularly multidrug resistant (MDR) and extensively drug resistant (XDR) TB cases. The majority of drug-resistant *Mtb* strains found in clinical settings emerge due to mutations in genes that are involved in the antibiotic mode of action (drug activator, drug target, etc.). These bacterial genes have important roles in bacterial metabolism and pathogenicity. Therefore, the study of drug-resistant *Mtb* strains has been evolving from the exploration of the associated genotype (i.e. specific resistance-conferring and compensatory mutation(s)) to the entire phenotypic impact that mutation events confer to the bacteria beyond the drug resistance feature. The study of the global drug-resistant phenotype in *Mtb* thorough comprehensive system biology approaches (such as genomics, proteomics, and lipidomics) is expected to reveal important aspects that will help TB researchers in the development of new anti-TB chemotherapies and overcome the current global challenges toward an effective TB control. This chapter will describe an overview of *Mtb* physiology and metabolic pathways as an important scaffold to understand the physiological changes that some *Mtb* strains (specific genotypes) undergo after acquiring resistance to the major anti-TB drugs: isoniazid (INH) and rifampicin (RIF) from a biochemical perspective.

2. Review of *Mtb* major metabolic pathways and cell envelope

Mtb physiology is a broad subject comprising *the study of the function and activities of this bacterium and its parts*. In this chapter, we will narrow the study of *Mtb* physiology to its major metabolic pathways and cell envelope, particularly in the context of drug resistance.

2.1. Major central metabolic pathways in *Mtb*

Mtb has the ability to use very variable carbon sources *in vitro* such as carbohydrates, alcohols, and lipids (including cholesterol and fatty acids) (reviewed in Ref. [1]). Similar to other representative species of the Actinomycetales order, *Mtb* possesses a predominant aerobic metabolism, with the genes encoding for enzymes of the main energetic metabolic pathways such as glycolysis, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway. Despite the genetic evidence of a complete TCA cycle in *Mtb* [2], there is no sufficient biochemical evidence to show the presence of all enzymatic reactions of the TCA cycle in *Mtb*. In fact, some researchers propose that TCA cycle in *Mtb* is not complete because this organism lacks the alpha ketoglutarate dehydrogenase (α-KDH) enzyme [3–5]. Instead, alpha ketoglutarate decarboxylase (α-KGD and Rv1248c) and succinic semialdehyde dehydrogenase (GabD1/2, Rv0234, and Rv1731, respectively) are proposed as the enzymes that catalyze the step from alpha ketoglutarate (α-KG) to succinate in *Mtb* TCA cycle under normoxic conditions.

Particularly, α-KGD catalyzes the production of succinic semialdehyde, which can then be converted to succinate by GabD1/2 [5]. Also, experimental evidence suggests that *Mtb* operates a reversed TCA cycle with the reduction of fumarate to succinate to maintain the membrane potential in the absence of oxygen [6].

Mtb also has the glyoxylate shunt which allows the bacteria to bypass some enzymes of the regular TCA cycle under specific metabolic conditions (hypoxia or growth on fatty acids as carbon source) [2]. Under anaerobic conditions, the enzyme isocitrate lyase (Icl) (which is proposed to be required for virulence [7]), together with the α-KG ferredoxin oxidoreductase are believed to complete the cycle effectively bypassing the conversions of α-ketoglutarate to succinate to fumarate [8].

Mtb has the ability to use enzymes in multiple metabolic pathways to prolong its survival, a feature that is known as metabolic plasticity. For instance, Icl not only participates in the glyoxylate shunt and the methyl citrate cycle but also protects *Mtb* from the oxidative stress generated by the treatment with isoniazid (INH), rifampicin (RIF), and streptomycin [9]. Another example is the dihydrolipoamide dehydrogenase (Lpd) that can act as the E3 component of the pyruvate dehydrogenase or can provide electrons to the dihydrolipoamide succinyltransferase (DlaT, previously known as SucB) or be part of the branched-chain keto acid dehydrogenase complex to metabolize branched-chain amino acids [9]. On the other hand, *Mtb* has pathways with redundant enzymes (that include a variety of isozymes) that can catalyze the same reaction, which guarantees that vital processes occur despite possible external or internal stresses. A good example of this is the fatty acid degradation or β-oxidation pathway, which suggest that *Mtb* not only has a high lipid catabolism activity, but also that this is crucial part of its own metabolism [1].

2.2. Lipid metabolism: β-oxidation and fatty acid synthesis

Lipid metabolism is a highly relevant physiologic process in *Mtb*, with more than 6% of the genome devoted to these reactions and almost 20% of the genome encoding for genes related to cell wall processes. Lipid metabolism is an important part of this chapter as some enzymes of the lipid biosynthetic pathway are the target of anti-TB drugs such as INH and ethionamide. Compared to *Escherichia coli*, *Mtb* possess five times more enzymes dedicated to lipid metabolism. *Mtb* lipid metabolism is more lipolytic than lipogenic, probably as a result of the wide variety and amount of lipid sources in the human host as well as in the bacterial envelope [2]. For this reason, the first part of this chapter will focus on fatty acid degradation with a subsequent description of recent findings regarding fatty acid synthesis. Fatty acid degradation is a key process in *Mtb* metabolism and can explain some of its metabolic plasticity, while fatty acid synthesis is crucial in the understanding mechanism of action of the previously mentioned anti-TB drugs.

2.2.1. Fatty acid degradation

Fatty acid catabolism in *Mtb* is a process of successive oxidations where the β-carbon of the fatty acid is oxidized to a carbonyl group (**Figure 1**). In this process, the main goal is the synthesis of acetyl-CoA and reduced cofactors (such as NADH, $FADH_2$) that can fulfill energy requirements in the cell and also intermediates that can serve as substrate for anabolic processes [2]. Specifically, odd-chain fatty acids produce acetyl-CoA while even-chain fatty acids

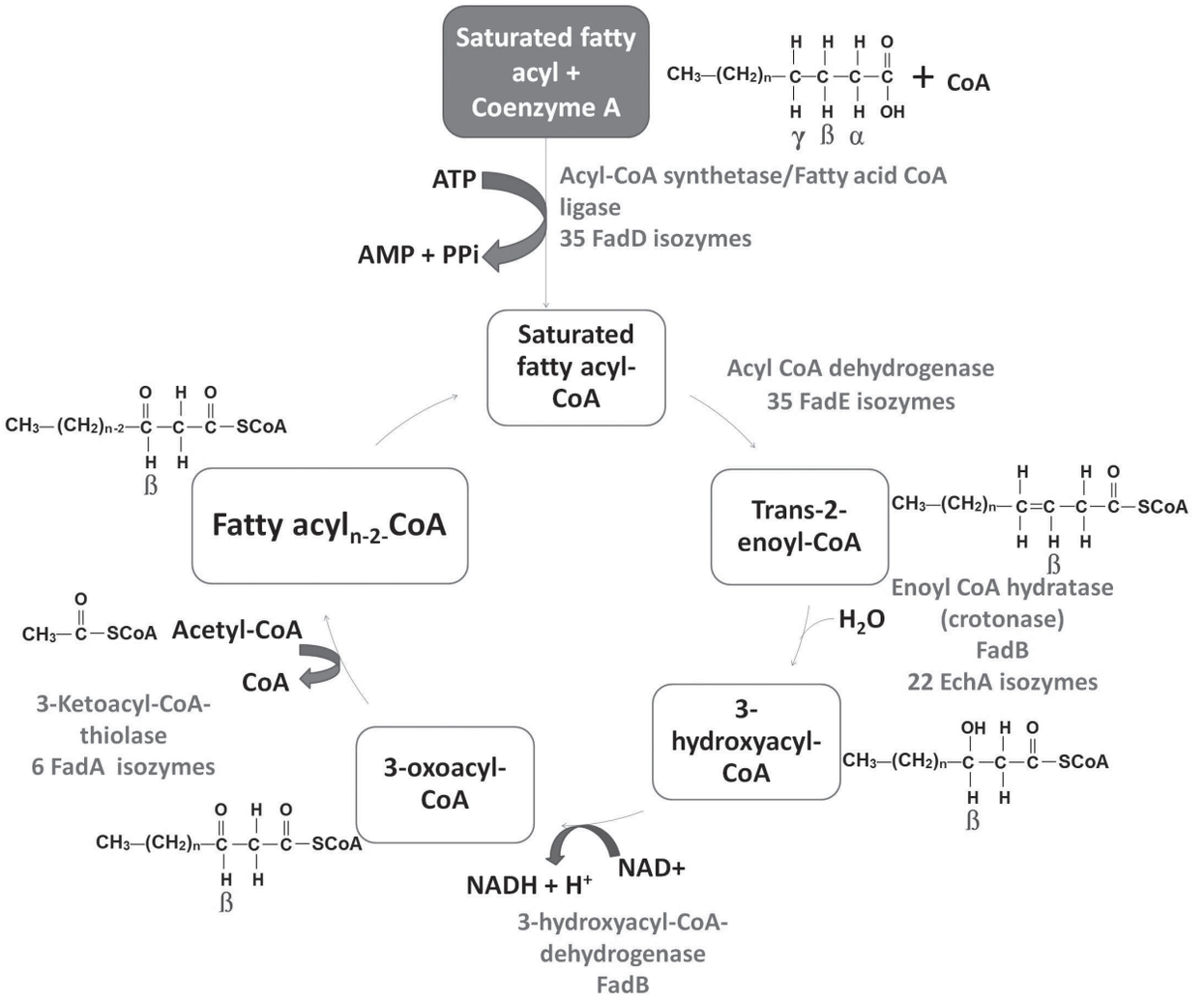

Figure 1. β-Oxidation of fatty acids in *Mtb*. Greek nomenclature indicates the different oxidations that take place in the β-carbon. The enzymes show the different number of identified isozymes that participate in this cycle.

produce acetyl-CoA and propionyl-CoA in addition to acyl-CoA derivatives missing two carbon units [2, 10]. By studying the *Mtb* genome, Cole et al. found at least 35 genes encoding for enzymes that catalyze the first step of fatty acid degradation only. As shown in **Figure 1**, most reactions in this pathway can be carried out by several isozymes. Of these, EchA5 and FadB3 are essential for *Mtb* growth and considered possible drug targets [11].

2.2.2. Fatty acid synthesis

The complexity of *Mtb* lipids can be partially explained by the fact that *Mtb* has both fatty acid synthases (FAS), type I and II. Cole et al. described the main enzymes of FAS I and II at the genetic level and recent reviews have compiled previous biochemical work, all of which have generated a better understanding of the complex pathways responsible for mycolic acid synthesis in *Mtb* [12–14]. The characterized enzymes that participate in FAS I and FAS II are shown in **Table 1**. FAS I is found mainly in eukaryotes and all the reactions are performed by a single multidomain homodimeric enzyme Fas (Rv2524) that has a mass higher than 300 kDa [2, 14]. This enzyme

Description	Gene	Rv number	Enzyme
FAS I	*fas*	2524	Fatty acid synthetase
Transition FAS I to FAS II	*fabD*	2243	Malonyl-CoA ACP transacylase
	accD6	2247	Acetyl/propionyl-CoA carboxylase (beta subunit)
	acpM	2244	Acyl carrier protein
	fabH	0533	β-Ketoacyl-ACP synthase III
FAS II	*kasA/B*	2245/2246	β-Ketoacyl-ACP synthase
	fab1 or MabA	1483	β-Ketoacyl-ACP reductase
	hadA/B/C	0635/0636/0637	(3)-hydroxyacyl-ACP dehydratase subunit A/B/C
	htdX	0241	3-hydroxyacyl-thioester dehydratase
	echA10/11	1142/1141	Currently annotated as a enoyl-CoA hydratase, but proposed to be 2-trans-enoyl-ACP isomerase
	inhA	1484	2-trans-enoyl-ACP reductase
Modifications			
Desaturases	*desA1/2/3*	0824/1094/3229	Acyl carrier protein desaturase
Methyltransferases (methylation, oxygen function introduction and cyclopropanation)	*mmaA1*	0645c	Methoxymycolic acid synthase 1
	mmaA2	0644c	Methoxymycolic acid synthase 2 (distal cyclopropane in α-MA, proximal cis-cyclopropane in keto-MA)
	mmaA3	0643c	Methoxymycolic acid synthase 3 (oxygenated MA)
	mmaA4	0642c	Methoxy mycolic acid synthase 4 (oxygenated MA)
	cmaA1	3392c	Cyclopropane-fatty-acyl-phospholipid synthase 1 (distal position)
	cmaA2	0503c	Cyclopropane-fatty-acyl-phospholipid synthase 2 (proximal position-specific in methoxy-MA)
Mycolic acid modification	*pcaA* (*umaA2*)	0470c	Mycolic acid synthase (proximal cyclopropanation function α-MA)
	umaA	0469	Mycolic acid synthase
Clainsen-type condensation	*accD4*	3799c	Acyl-CoA carboxylase
	accD5	3280	Acyl-CoA carboxylase
	fadD32	3801	Fatty-acid-AMP ligase
	pks13	3800	Polyketide synthase-13
Mycolic acid processing	*mmpL3*	0206	Transmembrane transport protein-3
	Rv3802	3802	Proposed to be a Mycolyltransferase I, recently shown to have phospholipase and thioesterase activity
	cmrA	2509	Reductase
	fbpA/fbpB/ fbpC2	3804c/1886c/0129c	Fibronectin-binding protein ABC or antigen 85 complex

Table 1. Enzymes that participate in the FAS I and II pathways in *Mtb*.

has seven catalytic domains: acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein, β ketoacyl reductase, and β ketoacyl synthase [12]. Fas (Rv2524) uses acetyl-CoA and malonyl-CoA as substrates for the synthesis of acyl-CoA derivatives of 16 and 18 carbon units which are in turn used for the synthesis of membrane phospholipids. FAS I route also produces an acyl-CoA derivative with 26 carbon units that becomes the short α-alkyl chain or α-branch of the mycolic acids. FAS I and II are connected by the synthesis of acyl-CoA derivatives with 20 carbon atoms that are used in the FAS II pathway as the starting molecule for the elongation of mycolic acids (reviewed in Ref. [14]).

There are important aspects to highlight regarding FAS I and II in *Mtb*. First, proteins FabD, AcpM, and FabH act in the transition between FAS I and FAS II, generating ACP derivatives (the substrate required for the FAS II pathway). Second, there are two known Claisen-type reactions occurring: one before the FAS II starts (responsible for the condensation of malonyl-ACP with acyl-CoA and catalyzed by FabH) and one shared with the polyketide synthase system (catalyzed by Pks13). The latter reaction generates a carbon-carbon bond between two activated fatty acids at the end of the mycolic acids synthesis. This second condensation takes the α-branch (produced through FAS I) and the longer meromycolate chain (produced through FAS I and II) to form a "pre-mature mycolic acid" [13, 14].

Regarding FAS II specifically, this pathway is involved in fatty acid elongation instead of *de novo* synthesis (contrary to what occurs in most bacteria, where FAS II has *de novo* synthesis capacity) [12]. *Mtb* needs to use both FAS I and II to generate its characteristic mycolic acids [13, 14]. Therefore, the study of mycolic acids synthesis is in fact a study of both FAS pathways in *Mtb*. In FAS II, there is one different enzyme for each specific step, allowing for various levels of regulation. Most of the core enzymes of FAS II are NADPH or NADH dependent and organized in different clusters distributed through the genome (**Figure 2** and **Table 1**). FAS II can be further divided into type I and type II elongation (E1 FAS II and E2 FAS II, respectively). Here, both types are catalyzed by the core proteins InhA, MabA, HadABC, and FabD, and elongation can be done by either KasA (E1) or KasB (E2). Despite the sequence homology between the condensases KasA and KasB, they are predicted to participate in two different stages during the FAS II pathway: KasA may catalyze the first elongation steps (E1-FAS II) while KasB might be involved in the later steps (E2-FAS II), ultimately producing full-length mycolates with more than 40 carbon units [12, 13]. A representation of matured α-mycolic acid is depicted in **Figure 2B**.

The meromycolate chain resulting from FAS II cycle can be "decorated" with chemical modifications such as cyclopropanations and methylations that are introduced before the second Claisen-type reaction occurs. These modifications can be at distal or proximal positions and are carried out by S-adenosyl-methionine (SAM)-dependent methyl transferases (**Table 1**). Unsaturations on the other hand, are proposed to occur differently under aerobic or anaerobic conditions. The method of double bond introduction in mycolic acid in *Mtb*, however, remains unclear. Under aerobic conditions, desaturases encoded by *desA1*, *2*, and *3* and other candidates such as Rv1371 are believed to complete the double bond introductions at the distal position, before the Claisen-type condensation take place. Under anaerobic conditions, unsaturations are believed to take place during the FAS II cycle in

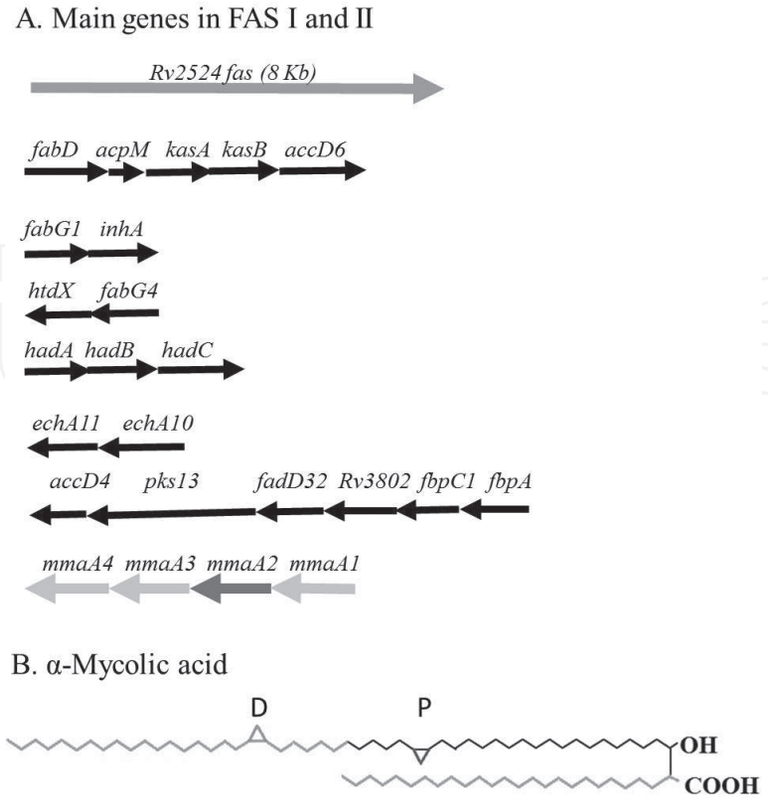

Figure 2. (A) Major operons involved in mycolic acid synthesis in *Mtb*. (B) Structure of an alpha-MA, the color represent the source of the carbon chain by either FAS I (light gray) or FAS II (black). P, proximal; D, distal.

the transition of the trans 2-enoyl intermediate to its 3-cis isomer in the distal position, resembling what FabM does in *Streptococcus pneumoniae* [13]. By sequence homology, this enzymatic reaction could be mediated by EchA10 and EchA11 in *Mtb*; however, there is not enough experimental evidence to support this hypothesis (**Table 1**). Finally, the oxygenated mycolic acids (keto and methoxymycolic acids) have a common precursor (hydroxymycolate) that is synthesized by the action of the SAM-dependent methoxymycolic acid synthase 4 (MmaA). The synthesis of methoxymycolic acid is additionally driven by the MmaA3 enzyme (**Table 1**) [12, 14].

After the modification in the meromycolate chain and the last condensation reaction occur, a mycolic acid (either α-, keto, or methoxymycolic acid) molecule is formed and can be attached to a trehalose molecule by the action of the Corynebacterineae mycolate reductase A, encoded by Rv2509 (also known CmrA) [15]. Once the mycolic acid is covalently linked with trehalose to form trehalose monomycolate (TMM), it is transported to the cell wall by the protein MmpL3 [16]. TMM is then the source of the mycolyl group for arabinogalactan and for other TMMs in the cell wall, generating trehalose dimycolate (TDM); in a reaction catalyzed by the fibronectin-binding proteins (Fbp) ABC ([17], reviewed in Refs. [14, 18]). Much of the understanding of the FAS I and II routes has been based on sequence homology with reference bacterial strains and mutation analysis using model organism such as *Mycobacterium smegmatis* and *Mycobacterium phlei*. Despite the vast knowledge about the mycolic acid synthesis pathway,

many unanswered questions remain regarding components of the FAS II pathway that are under current research [13].

2.3. Redox metabolism

In general, reduction-oxidation (i.e. redox) reactions are highly relevant for *Mtb*, since they not only comprise the necessary defence mechanisms developed to combat the host response during the infection, but they are also part of its own bacterial metabolism [19]. Redox reactions could generate endogenous or exogenous stress for the bacteria. The endogenous redox stress is generated during aerobic or anaerobic respiration, where *Mtb* is exposed to reactive oxygen (ROI) and reactive nitrogen intermediates (RNI), generated when the bacterium uses oxygen and nitrogen as the final electron acceptor in the electron transport chain, respectively [20, 21]. RNI can be also generated when *Mtb* relies on glutamate metabolism for survival. During host-infection, *Mtb* can experience a wide range of oxygen levels that can drastically alter its metabolism going from hyperoxic stress (when is in aerosol droplets) to low oxygen tension (during the intracellular phase in alveolar macrophages) to finally hypoxic to anoxic stress (in granulomas). Additionally, inside the macrophage, *Mtb* is exposed to both ROI and RNI. Hydrogen peroxide (H_2O_2) and the superoxide radical (O^{-2}) are the two most common ROI forms that are produced by macrophages and neutrophils to eliminate *Mtb* [22]. During hypoxic conditions, the alteration in redox homeostasis leads to a higher NADH/NAD^+ ratio which generate superoxide radicals that disrupt the redox balance in the cell. Consequently, enzymes with heme and sulfur complexes (i.e. cytochrome C, aconitase) can be severely affected. Therefore, the ability of *Mtb* to survive the redox stress from the host determines its success during the infection process. This stress has an impact on the bacterial metabolic pathways as well as on the expression of virulence factors [20, 21].

Intracellular or exogenously originated reactive oxygen species (ROS) and RNI have the potential to damage lipids, DNA, and proteins by oxidation, peroxidation, and nitration reactions [23], which can result in protein inactivation, and alteration of both cell organization and signal transduction. Therefore, it is crucial to successfully maintain redox homeostasis to keep the integrity of the cell. Intracellularly, the changes in the redox and nutrient levels are sensed by WhiB proteins (WhiB1-7) while extracellularly different molecules such as nitric oxide (NO), carbon monoxide (CO), and H_2O_2. The reduced and oxidized forms of the nicotinamide adenine dinucleotide (NADH/NAD^+) can work as sensors that induce a direct transcriptional response or indirectly alter transcription through a two-component regulatory system such as DosRS-DosRT [2, 21]. Moreover, different bacterial enzymes participate in the neutralization of the host-induced ROI and NOI such as superoxide dismutase (SodA), catalase-peroxidase (KatG), and the antioxidant complex formed by alkyl-hydroperoxidases (AhpC and AhpD), dihydrolipoamide acyltransferase (DlaT), and dehydrogenase (LpdC). Other enzymes in the redox metabolism include the peroxiredoxins (AhpE, TPx, Bcp, and BcpB) and thioredoxins (TrxA, B, and C).

Of these, KatG also plays a central role in *Mtb* resistance to INH. *Mtb* has only one single copy of *katG* with a coding sequence of 2223 base pairs (bp) generating a 704 amino acid protein with a molecular weight of approximately 80.6 kDa. KatG is presented as a dimeric haemoprotein that belongs to class I peroxidase superfamily, because of its high homology with yeast cytochrome C peroxidase [24]. KatG activates the prodrug INH, however its functions extends

beyond this activation. This enzyme is in fact one of the most important catalase-peroxidases that help the bacterium overcome external and internal redox stress. KatG possesses a monofunctional catalase, broad-spectrum peroxidase, and peroxynitritase activity [25, 26]. The catalase-peroxidase activity is in the N-terminal domain of the protein that contains a heme-binding motif, however, the C-terminus is also required for its catalytic function [24, 27]. KatG activity has also been associated with virulent *Mtb* strains, which are able to infect for longer periods and cause increased pathology in the host [28, 29].

As discussed above, redox reactions play an important role in bacterial respiration. In the next section, details about the cellular respiration process in *Mtb* are discussed. The relation with this topic and this chapter is based on the association of drug resistance mutations in important genes such as *katG*. The mutations in these redox homeostasis genes possibly generate an alteration in the respiration complexes in *Mtb* as well.

2.4. Respiration in *Mtb*

Given the dynamic *Mtb* lifestyle, respiration in the bacterium should be highly adaptable. Specifically, during respiration, *Mtb* uses oxygen and other compounds (such as fumarate or nitrate) as the final electron acceptor depending on the specific bacterial metabolic status and the surrounding environment [30, 31]. The respiratory apparatus is responsible for generating ATP and reduced coenzymes (NADH and/or $FADH_2$). Respiration is made possible by selected membrane-associated asymmetric complexes that allow for generation of proton motive force (PMF) and ATP, which are the major sources of energy in the cell. Different from other model organisms such as *E. coli* or *Bacillus subtilis*, *Mtb* obtains the majority of its ATP by the electron transport chain and the F_1F_0-ATP synthase machinery, with very little contributions from substrate level phosphorylation [31]. In fact, the ATP synthase is a recently successfully exploited target for developing anti-TB drugs of the drug class diarylquinolines such as the recently described clinical drug bedaquiline [31, 32]. Specifically, diarylquinolines interact with the transmembrane subunit C of the ATP synthase machinery [33]. This again emphasizes the importance of ATP synthase machinery in the respiration process in *Mtb*.

Most of the *Mtb* enzymes/complexes involved in aerobic respiration have been identified and are composed primarily of two NADH dehydrogenases (NDH-1 and NDH-2) and two terminal cytochrome oxidases (aa3-type cytochrome C oxidase and bd-type cytochrome oxidase). These enzymes participate in oxygen reduction and are coupled to generate the PMF that is used by the ATP synthase for the production of ATP. NDH-1 is encoded by the *nuo* operon (*nuoA-N*) and NDH-2 is present in two copies encoded by *ndh* and *ndhA*. Previous studies demonstrated that NDH-2 does not have a proton-translocating-function and is the main dehydrogenase in *Mtb*. NDH-2 reduces menaquinone to menaquinol that in turn can be oxidized by one of the terminal aa3-type cytochrome C oxidase and bd-type cytochrome oxidase complexes. Because the bd-type cytochrome oxidase (CytA-B) is not coupled to proton pumping, the direct oxidation of menaquinol by this oxidase is less energetically efficient compared to the aa3-type (CtaC-F). Instead, the oxidation of menaquinol can happen in a two-step process with the participation of the cytochrome bc1 complex (QcrA-C) and the terminal aa3-type cytochrome oxidase (CtaC-F) with a higher energy yield [31, 32].

Contrary to aerobic respiration, mediators in *Mtb* anaerobic respiration are poorly defined. However, *in vitro* hypoxic studies have allowed the identification of some important enzymes involved in this process. In a reduced-oxygen environment, the nitrate reductase (NarG-I), the nitrate transporter (NarK-2), and the NDH-2 dehydrogenase are upregulated. On the other hand, the ATP synthase subunits and the aa3-type cytochrome oxidase are downregulated. During a low oxygen tension, the bd-type cytochrome oxidase is believed to be more utilized since it has a higher affinity for oxygen. ATP synthase is still active although at a lower membrane potential not commonly seen in other organisms, underlining the importance of PMF in keeping the bacterium alive during this metabolic state. This could be a regular scenario for *Mtb* inside the granuloma driving low metabolic activity with low or no *Mtb* growth (dormancy) [22]. Also, in the absence of oxygen, *Mtb* uses a set of reductases (such as succinate/fumarate reductase and nitrate reductase), hydrogenases (coupling H_2 oxidation to respiration, encoded by Rv0082 and Rv0087), and ferredoxins (such as the encoded by *fdxA*) that preserve the PMF for bacterial survival [30, 31]. Other changes have been detected in anaerobic adaptation, for instance, the E1 subunit of the pyruvate dehydrogenase is upregulated. Under anaerobic conditions, *Mtb* can stay alive but its growth is strongly reduced [31]. This theme is relevant because as it was previously described, there is a wide variety of oxygen tension in the *Mtb* interaction with the host.

2.5. *Mtb* envelope

Moving to another important aspect of *Mtb* physiology, the cell envelope of this bacterium has been the focus of research for many decades because of its distinct features, importance in bacterial pathogenicity, and the generation of the host immune response. The mycobacterial cell envelope is complex such that nutrients penetrate 10,000 times slower than they can do in the *E. coli* outer membrane [34]. Components of the cell envelope, particularly the enzymes that participate in their synthesis, have been recognized as possible drug targets. The understanding of the cell envelope is also required to design drugs that will be able to cross this impermeable barrier efficiently [35].

The *Mtb* envelope forms the interface between pathogen and host. From the outside to the inside, the *Mtb* cell envelope is composed of a layer of non-covalently linked glycolipids, proteins, carbohydrates, and some lipids (the capsule), a covalently linked peptidoglycan layer that contains carbohydrates and lipids (the cell wall), and a plasmatic membrane (phospholipid bilayer). In 1991, Minnikin proposed visualizing the lipid material in the *Mtb* envelope as two distinct membranes, analogous to a Gram-negative bacterium [36].

The most external layer of *Mtb* has been described as a "capsule" by some scientists. This layer contains mainly polysaccharides and a small amount of lipids (2–3%). The major capsular component in slow growing mycobacteria, including *Mtb* is a glucan composed of repeating units of ->4-(-D-glucosyl residues substituted at position 6 with a mono- or oligoglucosyl residues). The capsular material also contains the heteropolysaccharide D-arabino-D-mannan and a mannan chain composed of ->6-(-D-mannosyl-1-> core with substitutions at some positions 2 with a (-D-mannosyl unit. Finally, the arabinomannan found in this extracellular material is "decorated" by other oligomannosides, which can be also secreted to the extracellular space [35], reviewed in

Ref. [37]. Glycolipids such as trehalose monomycolate (TMM) and trehalose dimycolate (TDM); phenolic lipids and glycopeptidolipids can be found in the outer part of the capsule and some of them are also cell wall-associated. TDM is also known as cord factor since it causes grow in "cords" *in vitro*. This particular glycolipid has been associated with the pathogenesis and immunogenicity of *Mtb* strains [38]. Lipoproteins such as LpqH (Rv3763), proteins such as Psts1 (Rv0934) and the Ag85 complex (FbpA, Rv3804; FbpB, Rv1886; and FbpC, Rv0129) are also commonly found in the capsular material [35].

The *Mtb* cell wall has a covalently linked backbone with a collection of cell wall-associated lipids and polypeptides. The covalently linked molecules include peptidoglycan, arabinogalactan, and mycolic acids. In addition to the presence of the last two biomolecules, there are two important hallmarks of the *Mtb* cell wall. First, the muramic acid is N-acylated, instead of N-acetylated as regularly observed in most eubacteria. Second, there are unusual cross-links between two chains of peptidoglycan that include bonds of two residues of diaminopimelic acid in addition to the usual D-alanyl-diaminopimelate linkage. Furthermore, mycolic acids represent about 40% of the cell wall [39, 40].

Mtb has a great variety of lipids that can be clustered into at least six lipid categories with around 2512 lipid groups [41]. Mycolic acids are the major constituent of the cell envelope. They were first named by Stodola and colleagues in 1938, who also depicted essential groups of their chemical structure. Mycolic acid structure was further defined by Asselineau in 1950 [42]. These are α-alkyl, β-hydroxyl, long-chain fatty acids that can be primarily covalently attached as esters of arabinogalactan in the cell wall or as "free lipids" in the capsule associated to trehalose in the TMM or TDM structures [12, 43]. Specifically, mycolic acids form an ester bound to the 5-position of the arabinose residue of the arabinogalactan [41]. Mycolic acids can also bind to glucose [44]. The covalently attached mycolic acids can be obtained by saponification or methanolysis of the cell wall of the delipidated *Mtb* cells. Because mycolic acids are not soluble in methanol, they can be separated from moderately long-chain fatty acids with ether or chloroform solutions. Mycolic acids have one carbon chain bound to the hydroxyl group called the meromycolic chain and another (shorter) carbon chain that is bound to the α-carbon [35]. The synthesis of these molecules was previously discussed in Section 2.2.2 of this chapter.

Mycolic acids are not unique structures of the *Mycobacterium* genera, they can be present in *Corynebacterium*, *Nocardia*, and *Rhodococcus*. Mycolic acids from *Mycobacterium* are longer in carbon units (C70–C90) and have the largest meromycolic chain [39]. Additional modifications such as the introduction of cyclopropane rings in the meromycolate chain, unsaturations, ethylenic groups, and methyl branches are also observed. Both *cis* and *trans* double bounds as well as cyclopropane rings can be found in the same type of mycolate. Some mycolic acids have additional oxygen functionality that is one feature used to classify them. These functionalities are keto, methoxy, carboxy, and epoxy. Other types of mycolic acids lack of these oxygen groups, they are called α-mycolic acids and α'-mycolic acids. α and α'-mycolic acids differ in their chain length, α'-mycolic acids are shorter (usually of 60 carbon units) whereas α-mycolic acids contains more than 70 carbon units. α-mycolic acids represent more than 70% of the total mycolic acids found in *Mtb*, followed by keto and methoxy variants (15 and 10%).

The cyclopropane structures in this fatty acids contribute not only to its cell wall structure, but also protect the bacteria from oxidizing agents such as H_2O_2 (reviewed in Ref. [14]).

Finally, the plasmatic membrane includes different types of phospholipids such as phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides (PIMs). PIMs are mainly located in the outer leaflet. Other important components are the highly immunogenic lipoglycan lipoarabinomannan (LAM) and lipomannan [39]. Due to the high abundance of LAM in the *Mtb* envelope, it has been tested as a biomarker for a point of care test with a wide range of sensitivity and specificity results in HIV-positive patients [45].

3. Mechanisms of drug resistance in *Mtb*

As is the case in other microorganisms, drug resistance in *Mtb* can be either intrinsic or acquired. *Mtb* cell wall structure and its low permeability are the major factors accounting for the high degree of intrinsic or natural tolerance to many antibiotics and other chemotherapeutic agents. Highly abundant mycolic acids in the cell wall reduce the cell permeability and create a crystalline-like structure after the cytosolic membrane. As seen in other mycobacterial species (especially in saprophytic species such as *Mycobacterium chelonae*), the more impermeable the cell wall, the more antimicrobial agents the mycobacteria can resist. Drugs such as sulphonamides, penicillin, tetracycline, and vancomycin are ineffective against *Mtb*. For vancomycin, this can explained because of its size and structure that do not allow its effective penetration through the *Mtb* "pseudo-outer membrane" [34]. However, recent findings demonstrated that *Mycobacterium bovis* and *Mtb* mutants lacking phthiocerol dimycocerosates are susceptible to glycopeptides such as vancomycin [46]. Additionally, the reduced number of porins in the *Mtb* "pseudo-outer membrane" possibly contributes to the intrinsic *Mtb* resistance against hydrophilic compounds. Among other intrinsic factors, *Mtb* possess β-lactamase enzymes (encoded by *blaC* and *blaS*) that make this bacterium naturally resistant to β-lactams [2, 47]. For acquired drug resistance, spontaneous mutations in chromosomal genes during a suboptimal drug therapy are the most common cause for drug resistance in *Mtb*. Efflux mechanisms are less common but also present in these bacteria [47]. These intrinsic and acquired mechanisms have synergistic effects and make TB treatment particularly cumbersome.

Although a combined therapy for TB is normally effective for most cases, TB cases resistant to a subsection or all anti-TB drugs have been reported in clinical settings. Because INH and RIF are the most widely anti-TB drugs used, there is a higher frequency of mono-resistance to any of these drugs or to both drugs (INH and RIF, known as multidrug-resistance TB or MDR-TB) among drug-resistant *Mtb* strains. The study of drug-resistant TB has been an ongoing process, mainly because the understanding of the mechanism of action of several first line drugs (such as INH and pyrazinamide) has been subject of intensive research and controversies [48]. The cumulative exposure of *Mtb* strains to suboptimal concentrations of anti-TB drugs in an intermittent manner creates most of the acquired drug-resistant TB cases. In this way, many TB patients lose the best options for effective treatment from a disease that was initially curable.

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3.1. INH resistance

In 1951, the anti-TB properties of a new drug, INH, were reported. This was a critical event in TB history that was optimistically described as the "new treatment for the white scourge." Unfortunately, the appearance of INH-resistant (INHr) cases emerged the same year INH was introduced in medical practice [49]. INH resistance is one of the most common forms of drug-resistant TB. The resistance mechanism to this drug is multigenic and can be divided into three categories: prevention of drug activation, alteration of the target, and differential expression of the target. In the first group, mutations in *katG* that prevent the activation of INH are present in the majority of resistant cases to this drug [50, 51]. *Mtb* strains with a full deletion of *katG* also fall into this category. KatG function was first correlated to INH resistance in 1953, when Middlebrook et al. discovered that INHr *Mtb* strains lacked catalase-peroxidase activity and were less virulent in guinea pigs [52]. The molecular validation of this observation was completed later by Zhang et al., restoring the sensitivity to INH in some *Mtb* resistant strains after the introduction of the *katG* gene from *E. coli* [53]. More than 60 years of chemotherapy with INH in TB cases has allowed the development of different *Mtb* genotypes of INH-resistant (INHr) profile and their associated phenotypes. Currently, there are more than 300 known mutations in the *katG* gene alone associated with a wide range of minimum inhibitory concentrations (0.2–256 mg/L) [51]. These mutations include missense mutations, insertions, deletions, truncations, and full gene deletion. Depending on the position and nature of the mutation, *katG* mutants have different degrees of catalase-peroxidase activity [47, 51]. The mutation rate for the generation of INH-resistant strains is around 3.2×10^{-7} mutations/cell division (after exposure to 1 mg/L INH) *in vitro* [54–56] and presumably one in 10^{8-9} organisms *in vivo* [57].

In the category of alteration of the target and increased expression of the target, mutations in the *inhA* gene or its promoter are accounted. InhA is the most commonly validated target for INH [49, 58]. Currently, around 15 mutations in the *inhA* gene have been identified in *Mtb* strains with low-level resistance to INH. *inhA* mutations also drive resistance to ethionamide (ETH), since INH and ETH share this enzyme as target [51]. The most studied mutation is the S94A that results in the reduction of the enzyme affinity for NADH and a reduced ability of INH-NAD adduct to inhibit the enzyme. Additionally, mutations in the *inhA* promoter that increase InhA levels have been also identified. Therefore, both the reduction in enzymatic activity, specifically KatG and the overexpression of the target (InhA) serve as resistance mechanisms against INH. Other mechanisms of INH resistance include the accumulation of NADH (by redox alteration) that binds InhA and protects it from the inhibitory effect of the INH-NAD adduct. An additional resistance mechanism includes acetylation of the drug by the *nat* encoded arylamine N-acetyl-transferase which prevents INH activation by KatG [59, 60]. Finally, the drug efflux mechanisms include the participation of the protein EfpA, which is induced upon INH treatment [49]. It is important to describe that INH resistance in *Mtb* can be either low- or high-level when there is >1% of bacterial growth in the presence of 0.2 or 1 μg/mL of INH, respectively. Regularly, mutations in the *inhA* promoter are linked to low-level of INH resistance while mutations in *katG* are associated with high-level of INH resistance in *Mtb* [61].

3.2. RIF resistance

Followed the discovery of INH, rifampicin (RIF) was discovered in 1963 and reduced the anti-TB treatment from 18 to 9 months [62–64]. Currently, a shorter combined therapy with higher doses of rifampicin or isoniazid is being evaluated [65]. The rationale behind the increase dose of rifampicin is that the currently used dose of RIF was proposed in 1971 with the basis of generating a cost-effective treatment that was non-toxic for TB patients, albeit a study of the maximum dose of the drug tolerated in human has never been performed [66]. Recent studies in animal models have shown that higher doses of this drug could be effective even in shorter regimes, reducing also the probability to generate resistant microorganisms to the drug [66–68].

RIF resistance in *Mtb* is simpler than INH resistance. Up to date, mutations in one gene, *rpoB*, that encodes for the RNA polymerase β subunit and the target of the drug, are present in most of the RIF resistant (RIFr) cases. There are only four in the *rpoB* gene (N-terminus, and clusters I–III) where most of these mutations are found. In fact, mutations in an 81 base pair (27 codons) in the central region of cluster I, also known as the RIF resistance-determining region (RRDR), harbors more than 96% of all mutations associated with RIF resistance. Similarly to what is described for INH resistance, these mutations can be single amino acid substitutions, deletions, and insertions [62]. These mutations mainly affect the binding pocket where the drug interacts with the subunit of the polymerase. The most common amino acid substitutions observed in clinical RIFr strains include S531L and H526Y [69].

4. Impact of drug resistance in *Mtb* physiology as seen through proteomics perspective

Since there is a wider repertoire of INH resistance-conferring mutations compared with RIF resistance-conferring mutations (see Sections 3.1.1 and 3.1.2), a more variable phenotype in INHr strains compared to RIFr strains is expected. Additionally, the genetic lineage and background of each strain play an important role in the phenotype resultant after drug-resistance is acquired [69–71]. This is explained by the fact that compensatory mutations associated with some genetic backgrounds but not others may results in different competitive phenotypes. Our laboratory recently demonstrated that the same mutation causing INH resistance in two *Mtb* strains from different genetic lineages can result in different virulent phenotypes. Furthermore, these differences were associated with differences in protein levels of AhpC without any detectable mutation in the *ahpC* gene or its promoter. These *Mtb* strains were from different genetic lineages and exhibited a strongly different virulent profile in the mouse model of infection [72]. Therefore, following a "conservative approach," comparing clinically relevant clonal or isogenic *Mtb* strains is crucial to understand the changes in *Mtb* physiology caused by drug resistance events. However, obtaining pure clonal pairs of *Mtb* derived from clinical settings is quite challenging.

Clonal *Mtb* pairs conceptually defines a pair or group of bacterial strains that share the same progenitor, but are generated after successive replication events with the possibility to develop

one or more single nucleotide polymorphisms (SNPs) each time, possibly due to external pressure such as drug exposure, oxygen tension among other factors [73]. The development of more discriminative and high-throughput genetic tools has allowed a more accurate characterization of these clonal and isogenic strains. Isogenic and clonal strains are difficult to obtain from clinical cases due to the possibility of infection with different clones of *Mtb*, especially in high burden TB countries such as India and South Africa [74]. Furthermore, most settings with high burdens of TB do not routinely perform whole genome sequencing and are not equipped to carry a biobank of *Mtb* isolates. In the next sections, we will explore specific examples of *Mtb* strains that experienced compensatory physiological events after acquiring INH and/or RIF resistance comparing them to their clonal or isogenic parental strain.

We have used comparative shotgun proteomics of different *Mtb* cellular fractions to describe different aspects of the *Mtb* physiology *in vitro* and *in vivo*, including the effects of drug resistance-conferring mutations in the new bacterial phenotype [75–82]. The advantage of evaluating differences in protein abundance at each cellular fraction allows confirming if any differences seen are due to a global redistribution of protein levels or if changes in protein abundance are instead associated with a specific compartmentalization of the protein. After the elucidation of the genome of many organisms, proteomics emerged as a powerful methodology that not only describes the sequence, structure, and function of the proteins, but also extends to the analysis of complex mixture of proteins using high-throughput techniques [83, 84]. Proteomics analyze mature proteins considering all the complex post-translational events that occur in the cell and that finally represent the bacterial phenotype. As it was stated by LaBaer in 2011, "proteins provide the verbs to biology" [85, 86], and proteomics allow for naming different biological events [87]. As the proteome of the cell variate parallel to internal metabolic variation and external cues, proteomics is considered the most direct scaffold to measure cell activity [86]. Mass spectrometry (MS)-based technologies are central components of the protein analysis. These methods include shotgun and targeted proteomics that have different modes for acquiring mass spectra. Shotgun proteomics, a term coined by John Yates III and his laboratory, offers an indirect measurement of proteins through peptides derived from their enzymatic digestion [84]. Shotgun proteomics, also known as discovery proteomics, uses liquid chromatography (LC) connected to tandem MS (MS/MS) for the identification of the protein components in the sample. The protein identification is based on the determination of the amino acid sequence which is achieved by comparing the experimental tandem mass spectra with the theoretical tandem mass spectra generated from an *in silico* digestion of a protein database.

4.1. "The isoniazid resistance case": findings from *katG* mutant *Mtb* strain of the Beijing lineage through proteomics

Given the high frequency of *katG* mutations among INHr *Mtb* strains, this section will focus on the proteomics findings that were revealed in the study of an isogenic pair of the Beijing lineage after acquisition of drug resistance due to a *katG* mutation [80]. As the starting point, it should be noted that early studies revealed that INHr *Mtb* strains with *katG* mutations have different levels of the enzyme and a different degree of alteration of its catalase or peroxidase

activities [88]. These mutations have also different impact in the virulence and fitness of the INHr bacterium. However, to our knowledge, the study described here is unique as it used clinical isogenic pairs of *Mtb* strains resulting from *katG* mutations and associated with an INHr profile.

Consistent with previous studies, the global proteomics study of the Beijing clinical pair through LC-MS/MS demonstrated that the INHr strain had significantly reduced levels of KatG in three of the four subcellular fractions evaluated compared with its isogenic INHs progenitor. The fact that the levels of this protein were reduced in the soluble fractions (cytosol and secreted proteins) and the bacterial membrane is a clear indication that this INHr strain lacks its ability to activate INH. An additional 45 proteins were found with altered abundance; these protein changes may be a potential compensatory mechanisms related to the reduced KatG levels and its consequent impact on mycobacterial physiology and fitness [80].

Among the 45 proteins identified, proteins related to intermediary metabolism and respiration represented majority of differentially abundant between INHr and INHs strains. Among them, enzymes from the tricarboxylic acid (TCA) cycle (SucC, SucD, Mdh, Acn, and AceE) were all decreased in the INHr strain. Proteins related to lipid biosynthesis and degradation pathways also represented important differences between the strains, with mainly higher levels in the INHr strain. The proteins Fas, FabG4, and FbpD of the lipid biosynthetic pathway were increased. In the β-oxidation pathway, the dehydrogenases FadE22 and FadE32 and the acetyl-CoA acyltransferase FadA2 were increased, but the crotonases EchA9 and EchA21 were decreased in the INHr strain. Proteins in the virulence and detoxification category such DnaK and GroES were also increased in the INHr strain as well as the hypothetical protein Rv2204c. Finally, the transcription regulation proteins Crp and PrrA were also higher in the INHr strain compared to the INHs parental strain [80].

Interestingly, the INHr Beijing strain had the *katG* mutation L101R (identified in the INHr by whole genome sequencing) [89]. However, this *katG* mutation was not very stable for the Beijing INHr strain, which after successive passes reversed to the wild type genotype and INHs phenotype. A previous report of an INHr reversion in *Mtb* was observed in a *katG* mutant in the absence of the drug pressure [90]. Based on these reports, it is possible that not only the resistant-conferring mutations can result in a distinctive phenotype but also that these mutations are not easily conserved in the *Mtb* genome after removing the pressure that originates them.

A previous proteomic analysis using non-clonal *Mtb* strains found five proteins overexpressed in the INHr strains comparing whole cell lysates. These proteins were found through two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption ionization time of flight-MS (MALDI-TOF) and include OpcA, FixB, RegX3, a probable oxidoreductase (Rv2971), and Wag31. Most of these proteins were involved in cellular metabolism, including redox metabolism (such as OpcA, Rv2971, and FixB) and there was one transcriptional regulatory protein (RegX3). These proteins are not related to any of the known INH-resistance mechanisms and were not observed in the previous clonal study. However, they still confirm the alteration of proteins involved in redox stress and energetic metabolism [91].

A recent virulence study of laboratory and clinical clonal pairs of *Mtb* from the T lineage and with different susceptibility profiles to INH also showed an important reduction of the KatG protein in the INHr strains. Associated with this KatG reduction, this study revealed a variable alkyl-peroxidase C (AhpC) response in the INHr strains which was dependant on the genetic background. Although both clinically and laboratory-derived INHr *Mtb* strains had reduced levels of KatG, western blot analysis with anti-AhpC demonstrated that the laboratory INHr strain had increased levels of AhpC while the clinical INHr strain had reduced levels of AhpC compared to their clonal parental strain, respectively. The difference observed in the AhpC levels was also translated in a non-significant reduction of the virulence in the laboratory INHr contrasting the strongly significantly reduced virulent profile for the clinical INHr strain [72]. A more robust proteomics study trough LC-MS/MS is being developed to reveal more insights about the proteomics differences among this clinical and laboratory-derived clonal pairs.

4.2. Acquisition of rifampicin (RIF) resistance in isogenic *Mtb* strains of the Beijing and Haarlem lineage

Phenotypic consequences of mutations in the *rpoB* gene associated with RIF resistance are understudied in *Mtb*. However in recent years, this theme has gained interest given the association of *rpoB* mutations with a variety of phenotypes in other microorganisms. For instance, in *E. coli*, *rpoB* mutations mimic the "stringent" response that is usually driven by ppGpp under stress conditions [92]. In *B. subtilis* and *Streptomyces coelicolor*, *rpoB* mutations are associated with an increased antibiotic production and increased production of other metabolites [93–95]. In *Neisseria meningitidis* and *Staphylococcus aureus*, *rpoB* mutations lead to a decrease permeability of the cell wall, which can be related to a subsequent increase in tolerance to certain antibiotics such as vancomycin [96–98]. Interestingly, after exposure to RIF, *Mtb* also appears to have an increased tolerance to ofloxacin, probably because of an increase activity of efflux pumps [99], although recent findings from our laboratory as well as others also suggest a potential role for cell permeability [75, 100]. In our study, isogenic *Mtb* pairs with two different *rpoB* mutations and representing two different genetic lineages (Beijing and Haarlem) showed an increased abundance of proteins involved polyketide synthesis. Proteomics findings were confirmed by an independent transcriptomics analysis of the strains grown intracellularly in *in vitro* macrophages. Both RIFr *rpoB* mutants revealed significant increased expression of multifunctional enzymes of the phenolpthiocerol synthesis type I polyketide synthase PpsE and C, which are involved in the biosynthesis of phthiocerol dimycocerosate (PDIM) and other lipids in *Mtb* [75]. We also observed a significantly increased abundance of the ABC transporter *drrA*, which has homology with other daunorubicin efflux pumps, but it is also implicated in export of PDIM across the cell membrane [101, 102]. Both increased abundance in lipids, as well as potential increase in efflux pump activity may result in accumulative reduction of cell permeability and may have important implications in subsequent acquisition of drug resistance.

4.3. Study of multidrug resistance in *Mtb* trough proteomics

A handful of proteomic studies focused on the comparison of drug susceptible (DS) versus multidrug-resistant strains (MDR) *Mtb* strains are available in the literature [70, 103–107].

Although these studies analyze clinical DS and MDR *Mtb* strains, the majority of them were comparing either non-related strains or strains with specific different genetic lineage. For instance, one study included H37Rv and H37Ra in the comparison and other compared DS Central-Asian (CAS)-2 with MDR East-African Indian (EAI)-3 strains [104]. The latter did not allow the study of the MDR phenotype under the same genetic background. There was one study that evaluated *Mtb* strains isolated from one single patient after many treatment failure episodes. Here, we will explore the findings specifically related to the DS and the first MDR strain isolated, since the next *Mtb* strains were also resistant to kanamycin. The MDR strain of the CAS 1-Delhi genotype had increased levels of 10 proteins through 2D electrophoresis and MALDI-TOF compared to its DS clonal pair. These proteins include chaperonin Hsp70, bacterioferritin BfrA, mycolyl-transferase FbpD, a component of the translational apparatus GatA, the phosphoserine aminotransferase SerC, Wag31, and the hypothetical proteins Rv1827, Rv2204c, Rv0543c, and Rv2004c [103]. Interestingly increased levels of FbpD and Rv2204c were also found in the INHr study of Beijing lineage [80]. Similarly, protein Wag31 was increased in a previous proteomic study monoresistant *Mtb* strains.

The proteomic analysis of non-genetically related *Mtb* strains revealed commonly increased levels of GroEL2, DlaT, ESAT-6, and conserved protein Rv3699 in the MDR strains compared to DS strains [70, 105–107]. Similar to the previous INHr proteomics studies mentioned above, some studies showed increased levels of FadA2, FabG4, BfrA, GroES, FixB, Rv2971, OpcA as well as lower levels of Mpt63 in MDR versus DS *Mtb* strains. However, there were contrasting levels of the proteins Mdh and SahH among the MDR studies and also discrepant tendencies of Fas in MDR strains compared to the INHr study of the Beijing genotype. On the other hand, there was one study that found increased levels of PpsC in a MDR strain compared to H37Rv as it was described in the RIF resistance proteomics study of isogenic pairs of Beijing and Haarlem genotype.

The analysis using non-genetically related strains provide valuable insights about the protein dynamics among DS and MDR *Mtb* strains. However, the fact that proteins such as the catalase-peroxidase KatG are increased in MDR strains without establishing the INH-resistance mechanism [104, 107], generates some questions such as: Is this protein increase because some genotypes express constitutively more KatG? According to this, it is not possible to conclude that there are actually INHr strains that have increased levels of KatG and highlight the necessity of study the drug resistance event under the same genetic background.

5. Lipidomics studies in *Mtb* drug-resistant strains

5.1. Lipidomics in INHr *Mtb* strains

Among the different scientific disciplines supporting biological research, metabolomics is the study of chemically diverse groups of biomolecules including sugars, nucleotides, peptides, lipids, among others; using technologies such as MS and nuclear magnetic resonance (NMR). Lipidomics is a branch of metabolomics that specializes on the water-insoluble metabolites—lipids. These are diverse metabolites that are part of the major molecules in the cell (particularly, in the cell membrane) [108]. In *Mtb*, lipids are a very relevant group of molecules, since

at it has been previously discussed, they are responsible for the intrinsic-drug-resistant nature against some antibiotics, and its synthesis has been the target of some anti-TB drugs (INH and ETH). Consistent with this idea, it is plausible to think that the study of the *Mtb* lipid is an important part of the description of drug-resistant *Mtb* strains.

Thus far, only one metabolomics study has been reported comparing *katG* mutant-INHr strains derived from a drug susceptible parental strain of the Haarlem genotype. Through 2D-gas chromatography-TOF MS, this study showed increased levels of saturated fatty acids (FA) in the INHr strains; particularly saturated FA with 16–20-carbon chain compared with its wild type that could be as a result of the oxidative stress which makes the bacteria rely on the β-oxidation of fatty acids as a carbon and energy source [109].

5.2. Lipidomics in RIFr *Mtb* strains

The lipidomics studies in RIF resistant *Mtb* have been focused on the RIF resistant strains that are both laboratory and clinically isolated. The laboratory-derived W-Beijing and CDC1551 were used as the parental DS strains and were exposed to 2 ug/mL RIF to select for the RIFr strains. In this way, three different *rpoB* mutants (S531L, Q513E, and H526Y for each *Mtb* strain) were studied. The analysis revealed reduced levels of di-acylated sulfoglycolipid (Ac2SGL) and mycobactins (including carboxymycobactins), while increased levels of PDIM compared to their DS parental strain. These compounds were identified among 172 features in the W-Beijing group and 102 features in the CDC1551 group analyzed by high performance liquid chromatography (HPLC) mass quadrupole-time-of-flight (QTOF) MS and suggesting a global remodeling of the cell wall after acquisition of RIF resistance [100]. This study supports previous findings from our group, which included a significant increase of diacylglycerol phosphocholines and PDIM precursors as observed by ultra-performance liquid chromatography (UPLC)-QTOF [75].

6. Closing remarks

The systematic study of *Mtb* phenotype, its proteome and metabolome (including, but not limited to lipidome) permits a functional description of how *Mtb* adapts, and sometimes thrives, under intrinsic (i.e. host response) and extrinsic pressure (i.e. exposure to drugs). These types of studies help to resolve not only the features of drug-resistant strains, but also contribute to the discovery of the facile and specific detection of biomarkers of drug resistance and ultimately contribute to the discovery of new targets for these *Mtb* strains that are hard to eliminate and often result in poor clinical outcomes for those infected.

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