Comparing the Metabolic Capabilities of Bacteria in the *Mycobacterium tuberculosis* Complex

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Abstract: Pathogenic mycobacteria are known for their ability to maintain persistent infections in various mammals. The canonical pathogen in this genus is *Mycobacterium tuberculosis* and this bacterium is particularly successful at surviving and replicating within macrophages. Here, we will highlight the metabolic processes that *M. tuberculosis* employs during infection in macrophages and compare these findings with what is understood for other pathogens in the *M. tuberculosis* complex.

1. Introduction

Mycobacterium tuberculosis is the causative agent of human tuberculosis (TB) and this bacterium is a member of the closely related cluster of species termed the *Mycobacterium tuberculosis* complex (MTBC). Members of the MTBC cause TB or TB-like disease in humans, livestock, and wildlife with high levels of morbidity and mortality. It is thought that all MTBC pathogens evolved from a common ancestor yet the bacteria fall into phylogenetically distinct lineages and clades under the following species names: *M. tuberculosis, M. africanum, M. microti, M. bovis, M. suricattae, M. mungi, M. orygis, M. caprae, M. pinnipedii,* and *M. canettii*.

Human-adapted MTBC isolates are categorized into seven distinct lineages. *M. tuberculosis* causes the majority of clinically observed TB cases in humans and is divided into five sub-lineages termed L1-L4 and L7. *M. africanum* causes TB in humans and comprises lineages L5 and L6 [1]. *M. microti* infects voles and occasionally causes TB in immunocompromised humans [2,3]. *M. bovis* can cause TB in humans, wild/domesticated bovines, possums, badgers, cervids, and goats [4]. Humans are considered dead-end hosts for *M. bovis* since human-to-human transmission with *M. bovis* is rarely observed [5]. *M. suricattae* is a meerkat isolate [6,7] and *M. mungi* is a banded mongoose isolate [8]. *M. orygis* has a broad host range including humans [9,10] and *M. caprae* is associated with goats [4]. *M. pinnipedii* is an isolate of seals, sea lions, tapirs, camels, and human animal trainers/workers [11–14]. Lastly, *M. canettii* is an environmental isolate that causes rare and sporadic, TB-like disease in humans [15,16].

These major lineages in the MTBC are primarily defined by small deletion mutations and these mutations are termed regions of difference or RD [17–22]. For example, *M. tuberculosis* H37Rv has 14 regions of difference (RD1–14), ranging in size from 2 to 12 kb, and this genetic material is deleted from the genome of *M. bovis* BCG-Pasteur [20,21,23]. The human-adapted strains, *M. tuberculosis* and *M. africanum*, are differentiated by the RD9 mutation that removed two genes in this region from the *M. africanum* genome [24]. MTBC isolates that rarely infect humans carry the RD9 deletion and lack genetic material within the RD7, RD8, and RD10 regions.

Genome comparisons across the *M. tuberculosis* lineages indicate that specific genes may be under different patterns of selection based on their putative function [25–27]. Recent findings indicate

that genes associated with metabolism in *M. tuberculosis* are under strong selective pressure in an experimental mouse model of infection [28]. This likely reflects the unique challenges associated with the facultative intracellular lifestyle of MTBC pathogens [29–37]. To replicate in macrophages, *M. tuberculosis* employs a specialized metabolism to counter nutrient limitations and the intrinsic immune pressures of macrophages [37]. Features of this specialized metabolism in *M. tuberculosis* are conserved among the MTBC pathogens and this is likely a unifying trait that contributes to the successful colonization of mammalian hosts [38]. Here we will review our current understanding of *M. tuberculosis* metabolism and discuss the metabolic similarities and differences that exist across this important group of pathogens.

2. Pathogenic Processes of the Canonical Pathogen, *Mycobacterium tuberculosis*

Human beings are the only natural host and reservoir for *M. tuberculosis*. A defining aspect of this disease is that the bacterium infects many, but causes a debilitating disease in relatively few individuals. In the year 2017 alone, *M. tuberculosis* caused an estimated 10 million new infections and was responsible for 1.6 million deaths [39]. The majority (>90%) of healthy individuals develop asymptomatic TB disease where the immune system contains but may not eliminate the bacteria. Co-morbidities, such as HIV infection that weaken immunity increase the potential for active TB disease, and roughly −10% of immuno-deficient individuals infected with *M. tuberculosis* develop active TB each year [40]. Key to the life cycle of *M. tuberculosis* is the bacterium's ability to persist and avoid clearance by host immunity between cycles of aerosol transmission.

Aerosol transmission deposits *M. tuberculosis* in the airways, where the bacterium infects alveolar macrophages, monocyte derived macrophages, dendritic cells, and neutrophils [41,42]. These infected cells stimulate expansion and/or recruitment of additional phagocytes and the eventual recruitment of antigen-specific T lymphocytes to the site of infection [43–45]. Antigen specific T lymphocytes exert immunologic control over the infection leading to a reduction in bacterial proliferation and limiting dissemination. The chronic nature of the infection promotes tissue damage and formation of granuloma lesions. Typically, TB granulomas are stratified lesions comprised of a necrotic caseous center surrounded by cellular layers enriched in phagocytes and lymphocytes [46]. Within these lesions, *M. tuberculosis* is typically observed in the acellular necrotic debris and/or internalized within phagocytic cells [47,48].

M. tuberculosis spends much of its life cycle intracellularly within macrophages and the bacterium avoids elimination within these cells by actively manipulating intracellular trafficking processes [49]. *M. tuberculosis* replicates in macrophage phagosomes do not fully acidify and are maintained at a pH of −6.4 [50–52]. These phagosomes maintain vesicular connectivity within the cell, receiving cargo from sorting/recycling endosomes [53] and the secretory pathway [51,54]. In resting macrophages, *M. tuberculosis* accesses nutrients to support bacterial growth within phagosomes [55–59]. Immunologic activation of macrophages with interferon gamma (IFN-γ) disables *M. tuberculosis's* ability to manipulate intracellular trafficking events, and the bacterial phagosome transforms into a restrictive compartment resembling a hydrolytically active acidified lysosome [60,61]. Activated macrophages do not completely eliminate *M. tuberculosis*, but these cells effectively constrain intracellular replication of the bacteria by limiting nutrient availability, producing nitric oxide and antimicrobial peptides, and autophagy-mediated processes [62–65]. Thus, even in the context of this single cell type, *M. tuberculosis* is subject to various constraints associated with nutrient availability and immune pressures imposed by the macrophage [64,66].

3. The Metabolic Capabilities of *M. tuberculosis*

Some intracellular pathogens are naturally auxotrophic and depend on macrophages to supply particular nutrients [67,68]. In contrast, *M. tuberculosis* is a prototroph and is equipped with the complete catabolic and anabolic pathways required to convert basic organic precursors into most, if not all, essential products (amino acids, co-factors, vitamins, nucleotides) [69]. *M. tuberculosis* also

simultaneously metabolizes multiple distinct nutrients *in vitro* [70] and in macrophages [71]. This metabolic flexibility likely allows *M. tuberculosis* to survive under various different nutritional stresses the bacterium encounters across its lifecycle. Therefore, nutrient abundance or availability along with pressures imposed by macrophages likely define some or all metabolic capabilities of *M. tuberculosis* at any given time. Due to the intimate interactions that occur between *M. tuberculosis* and the macrophage, we will focus on the bacterium's metabolism in this cell type and discuss themes that span across MTBC human and animal pathogens.

4. Carbon Metabolism

It has long been understood that *M. tuberculosis* preferentially utilizes host fatty acids *in vivo* [72]. Studies conducted over the past twenty years have all concluded that fatty acids are important carbon sources for *M. tuberculosis* during infection in macrophages [56,57,66,73–75]. More recently, it has become clear that cholesterol plays an equally important role in *M. tuberculosis* metabolism during macrophage infection [57,64]. *M. tuberculosis* uses fatty acids and cholesterol to fuel aneplurotic and energy producing reactions of central metabolism. Additionally, these substrates are used to synthesize bacterial lipid virulence factors that antagonize the immune response, promote pathogenicity, and facilitate persistence [76].

Bacterial degradation of cholesterol is unusual and relatively few bacteria are known to metabolize this molecule [77,78]. Saprophytic Mycobacteria subsist on dead or decaying organic matter in the environment and the ability to metabolize phytosterols or cholesterol from plant matter or animal carcasses likely provides a growth advantage in the environment [79]. The cholesterol metabolic pathway is conserved across the MTBC, and a recent genome comparison study predicted that a functional pathway is present in 51 of the 93 mycobacterial species examined [80]. Cholesterol is a mammalian cell membrane lipid, which macrophages acquires through endocytosis [81] and sequesters following inflammation [82]. It is likely that the ability to metabolize cholesterol provided an evolutionary advantage allowing an ancient MTBC bacteria, permitting colonization and survival in macrophages of mammals.

M. tuberculosis can metabolize carbohydrates *in vitro*, but the bacterium utilizes these carbohydrates to sustain homeostasis and/or biosynthetic reactions rather than energy production [83,84]. During infection, *M. tuberculosis* primarily synthesizes carbohydrates through the gluconeogenic conversion of lipid- and/or amino acid-derived intermediates [85]. Inactivating the gluconeogenic enzyme PckA in *M. bovis* BCG or *M. tuberculosis* restricts bacterial growth in macrophages [86,87]. Other bacteria in the MTBC likely synthesize carbohydrates via gluconeogenesis. MTBC isolates that rarely infect humans and strains and the human adapted *M. africanum* lineages L5 and L6 have a dead-end glycolysis pathway due to an E200D substitution in the pyruvate kinase enzyme, PykA [1,88]. PykA catalyzes the final step in glycolysis, and this mutation prevents the bacteria from growing on carbohydrate substrates. This mutation explains why pyruvate rather than glycerol has classically been needed to culture certain animal adapted isolates in the lab (Figure 1) [89].

MTBC bacteria also have increased flexibility around the pyruvate node in metabolism and can convert pyruvate into phosphoenolpyruvate (PEP), which may be important when the bacteria metabolize cholesterol. Cholesterol degradation produces excess pyruvate and this intermediate can fuel gluconeogenesis through the production of PEP via pyruvate phosphate dikinase, PpdK activity (Figure 1) [90]. While subtle differences exist across the MTBC, the overwhelming evidence indicates that all of these bacteria primarily synthesize carbohydrates via gluconeogenesis and preferentially utilize lipids and/or amino acids to fuel central metabolic pathways.
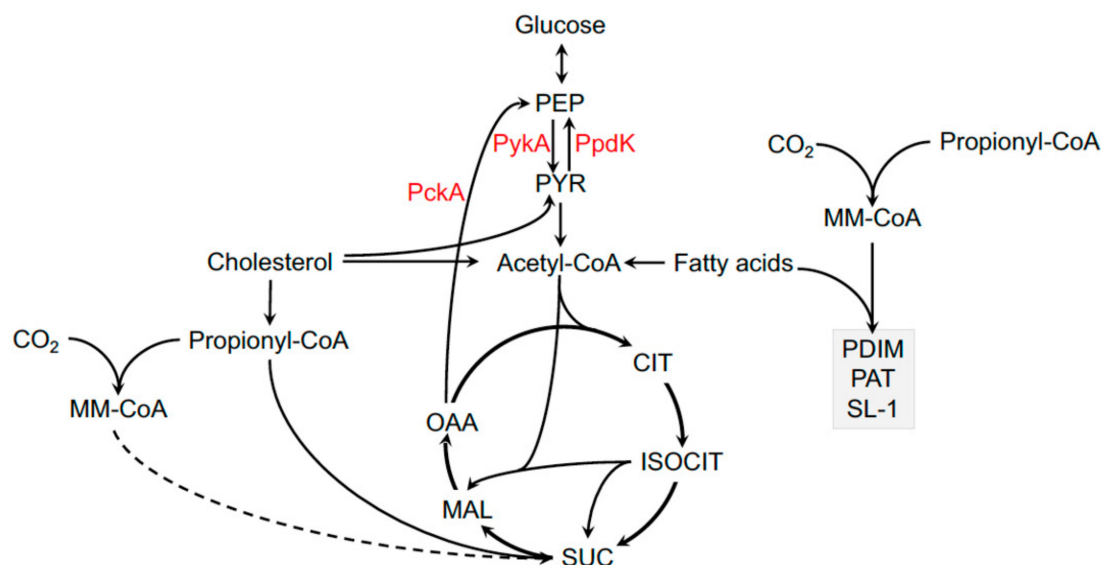
Figure 1. Central metabolic pathways of *Mycobacterium tuberculosis*. Central metabolic enzymes discussed in text indicated in red and methyl-branched polyketide lipids indicated in grey box. PEP, phosphoenolpyruvate; PYR, pyruvate; CIT, citrate; ISOCIT, isocitrate; SUC, succinate; MAL, malate; OOA, oxaloacetate; MM-CoA, methylmalonyl-CoA; PDIM, phthiocerol-dimycocerosate; PAT, polyacyl-trehalose; SL-1, sulfolipid; PckA, phosphoenolpyruvate carboxykinase; PykA, pyruvate kinase; PpdK, pyruvate phosphate dikinase.

5. Lipid Import

M. tuberculosis uses Mce transporters to import lipid substrates across the bacterial cell wall [76]. The *M. tuberculosis* genome encodes four separate Mce transporters termed Mce1-4 and it is known that Mce4 imports cholesterol [57,64] while Mce1 imports fatty acids [57,73]. These transporters are encoded individual operons within the *M. tuberculosis* genome and have long been implicated in the virulence of *M. tuberculosis*, particularly to promote bacterial growth in macrophages [91]. *M. tuberculosis* mutants lacking Mce4 replicate poorly in IFN-γ activated macrophages and have a slow growth phenotype in chronic infected mice [64]. It is unclear if this slow growth phenotype is due to restricted bacterial access to cholesterol, or an increased demand by the bacterium for cholesterol under conditions of IFN-γ activation. Expression of the Mce4 transporter varies across *M. tuberculosis* and is highly expressed by isolates from lineages L1 and L6 during infection in macrophages [92]. Proteins of the Mce1 transporter have long been considered virulence factors and are highly conserved across members of the MTBC [91].

The genes encoding the Mce1 transporter are considered core genes in the MTBC bacteria [93,94]. Informatic predictions suggest that *M. africanum* isolates in lineages L5 and L6 may carry a mutation in the *Mce1B* subunit but it is unknown if this mutation impacts Mce1 transporter activity [95]. Horizontal gene transfer is known to occur between environmental mycobacteria [96]. This process is thought to have been widespread in saprophytes prior to speciation in mammals [97]. Environmental mycobacteria commonly transfer genes to metabolize: 1) Amino acids and derivatives; 2) carbohydrates; 3) cofactors, vitamins, prosthetic groups, and pigments; and 4) lipids [98]. Interestingly, *M. chubuense*, a non-pathogenic environmental isolate, carries a plasmid with an intact Mce1 operon embedded in a transposon, suggesting that the ability to import fatty acids can be mobilized between mycobacterial species in the environment [98].

The substrates of the Mce2 and Mce3 transporters in *M. tuberculosis* remain unknown, but it is likely that these protein complexes are also transporters of hydrophobic nutrients. In the context of MTBC evolution, it is noteworthy to indicate that MTBC isolates that rarely infect humans and *M. africanum* lineage L6 lack the Mce3 transporter due to an eight-gene deletion associated with the RD7 mutation [19]. It is likely that this RD7 mutation prevents the import and metabolism of a yet-unknown

hydrophobic substrate that these pathogens do not require. Defining the substrate imported by Mce3 in *M. tuberculosis* will shed light on one major metabolic difference across the MTBC.

6. Fatty Acid Metabolism in *M. tuberculosis*

Fatty acids are a versatile carbon source for *M. tuberculosis*. The bacteria can -oxidize fatty acids to fuel gluconeogenesis and energy-producing central metabolic pathways or *M. tuberculosis* can use fatty acids for biosynthesis. *M. tuberculosis* shunts fatty acids to polyketide synthases for elongation into methyl-branched virulence lipids [99] or mycobactin to scavenge iron [100]. Fatty acids can also be elongated to form cell wall mycolic acids, or incorporated into membrane phospholipids [101]. Furthermore, fatty acids can be converted into triacylglycerol, which is thought to function as a carbon reserve, which can be metabolized when nutrients are limiting [102,103]. Similarly, *M. bovis* incorporates fatty acids as biosynthetic precursors to produce lipid-based end-products [104–108]. While the fate of fatty acids in the metabolism of animal-adapted MTBC remains poorly characterized it is expected that these processes are highly conserved across the MTBC members given the closely related lipid end-products these bacteria produce.

7. Cholesterol Metabolism in *M. tuberculosis*

Unlike fatty acids, it appears that cholesterol is degraded by *M. tuberculosis* exclusively to release acetyl-CoA, propionyl-CoA, and pyruvate all of which, fuel central metabolic pathways in the bacterium [76]. It is now appreciated that this cocktail of metabolic intermediates lies at a critical axis in *M. tuberculosis* metabolism that fuels energy-producing metabolism, gluconeogenesis, and biosynthetic pathways (Figure 1). The cholesterol-derived intermediate, propionyl-CoA, feeds into central metabolism via the methyl-citrate cycle (MCC) [109,110] or the methyl-malonyl pathway (MMP) [111]. Propionyl-CoA is also used to synthesize methyl-branched polyketide lipids (Figure 1) [99]. The unique flexibility of propionyl-CoA in *M. tuberculosis* metabolism allows the bacteria to sustain central metabolic pathways and balance toxic effects associated with cholesterol utilization (see below) [76,112,113].

The MCC assimilates propionyl-CoA into central metabolism via succinate and pyruvate, which occurs in a vitamin B12 (B12)-independent manner. In contrast, the MMP assimilates propionyl-CoA as succinyl-CoA in a B12-dependent manner (Figure 1). Bacteria and archaea generally synthesize B12, whereas humans and animals absorb most of this essential vitamin from their diet [114]. *M. tuberculosis* is not known to produce B12 *in vitro* or in macrophages suggesting the bacteria assimilates B12 or a B12 precursor directly from the host [115]. The soil dwelling bacteria, *M. canettii*, has every gene required to synthesize B12 while human- and animal-adapted isolates in the MTBC lack the B12 biosynthetic enzyme, CobF [116]. Thus, the inability to fully synthesize B12 may represent a specific patho-adaptation by members of the MTBC that reflects an increased dependence on B12 obtained from mammalian hosts.

Cholesterol degradation produces excess propionyl-CoA, which induce a metabolic toxicity in *M. tuberculosis* if the bacterium is unable to properly assimilate this intermediate [56,113]. Shunting propionyl-CoA into the biosynthesis of lipids can minimize the toxicity [56]. Through the biosynthesis of methyl-branched lipids, propionyl-CoA is assimilated as methyl-branched subunits in phthiocerol-dimycocerosate (PDIM), polyacylated trehalose (PAT), and sulfolipid (SL) [99]. During infection, *M. tuberculosis* produces excess PDIM and SL as a result of coupling cholesterol metabolism to the synthesis of these lipids [117–119]. It is currently unknown if this metabolic coupling links the production of virulence factors to an environmental condition or if this phenomenon simply reflects the excess amount of cholesterol that is metabolized by *M. tuberculosis* during infection.

PDIM has been implicated in macrophage invasion and recruitment [120,121], resistance to immune mediated stress [122–124], masking cell wall antigens [121], and facilitating bacterial escape from macrophage phagosomes [125,126]. All of these activities could be influenced by increased production of PDIM and/or other cell wall polyketide virulence lipids. Analysis of bacterial gene expression of MTBC lineages found that *M. africanum* isolates from L6 represses genes associated with

PDIM synthesis during infection in macrophages [92]. The majority of human- and animal-adapted MTBC strains produce a modified variant of PDIM that contains a phenolic glycolipid moiety, while most laboratory and European strains of *M. tuberculosis* do not produce this modified PDIM due to a mutation in pks15/1 [127]. The glycosylation patterns on this lipid vary across the MTBC [128], and this feature has been proposed to alter pathogenesis [129] and/or the immune response to this lipid [130,131].

8. Coupled Metabolism of Fatty Acids and Cholesterol in the Macrophage

Recent studies have revealed a "codependency" of fatty acids and cholesterol in *M. tuberculosis* metabolism. As mentioned above, cholesterol metabolism is associated with a metabolic toxicity and *M. tuberculosis* mitigates this by synthesizing methyl-branched polyketide lipids. Importantly the bacterium's ability to synthesize methyl-branched lipids is limited by the amount of available fatty acid primers. For example, cholesterol-mediated toxicity in an *M. tuberculosis* mutant lacking Icl1 is reversed by supplying excess fatty acids to infected macrophages and these fatty acids become incorporated into PDIM [56]. Thus, *M. tuberculosis* likely co-metabolizes fatty acids and cholesterol such that fatty acids are available as a "sink" for excess propionyl-CoA assimilation in the form of methyl-branched lipids such as PDIM. Thus, an important aspect of *M. tuberculosis* metabolism is to balance fatty acid and cholesterol utilization during infection.

9. *M. tuberculosis* Metabolic Flexibility and Macrophage Heterogeneity

Macrophages develop from distinct cell lineages and evidence is accumulating suggesting that developmental origin influences how these cells ultimately respond to infection and injury [132,133]. In mice, *M. tuberculosis* primarily resides in bone marrow derived, interstitial macrophages and, within alveolar macrophages that arise from the fetal liver during embryonic development [134]. The interstitial macrophages display an M1-like activation phenotype while the alveolar macrophages display an M2-like activation phenotype [134]. Additionally, in non-human primates, *M. tuberculosis* also resides in macrophages that express the M1 and M2 activation markers [135].

Importantly, these murine macrophage subsets have highly polarized metabolic states, where the M1-like macrophages are actively glycolytic and the M2-like macrophages are actively undergoing fatty acid oxidation [134]. This suggests that the types, and amounts of nutrients available to *M. tuberculosis* during infection vary with the host cell and the flexible metabolism of the bacterium may allow adaptation and survival within different macrophage cell types [134]. It is possible that in M2-like alveolar macrophages, *M. tuberculosis* may benefit from the increased import of lipids by the macrophage [134]. Perhaps in M1-like interstitial macrophages, *M. tuberculosis* may preferentially metabolize glycolysis end products such as lactate that accumulate in these cells [37,136]. It is currently unclear how *M. tuberculosis* fuels energy producing pathways in M1-like macrophages but *ex vivo* studies with human monocyte derived macrophages have demonstrated that *M. tuberculosis* can sustain a gluconeogenic pathway by assimilating lactate [137]. Going forward it will be interesting to understand how the metabolism of MTBC pathogens differs in specific host cells.

10. Nitrogen from Amino Acid Metabolism in *M. tuberculosis*

All cells require nitrogen to synthesize amino acids, nucleotides, and various essential cofactors. Recent studies have begun to shed light on how *M. tuberculosis* assimilates and metabolizes nitrogen during infection in macrophages. When cultured *in vitro*, *M. tuberculosis* demonstrates a preference for amino acids as a source of nitrogen over ammonia chloride, and *M. tuberculosis* also has the ability to co-metabolize multiple amino acids simultaneously [138]. During infection in macrophages it is thought that *M. tuberculosis* encounters nitrogen in two forms: As a nitrate derived from NO and as individual amino acids. In activated macrophages, *M. tuberculosis* can reduce nitrate (NO_{3-}) to nitrite (NO_{2-}) through the activity of the multi-subunit nitrate reductase, NarGHJI [139,140]. By using nitrate as an electron acceptor the bacterium can maintain respiration or cellular homeostasis when

oxygen is not available or limiting [141]. *M. tuberculosis* can also assimilate nitrogen from amino acids and when infected macrophages are pulsed with ^{15}N-labeled amino acids, both aspartate and asparagine accumulate in the bacterial phagosome and the bacteria require these amino acids to maintain infections [142,143]. Additionally, metabolic tracing experiments in macrophages labeled with ^{13}C-glucose established that a portion of the ^{13}C-label accumulates in host cell amino acids and when these cells are infected *M. tuberculosis* is able to scavenge the ^{13}C-labeled amino acids [71].

Nitrate reductase activity has been used for over 50 years in diagnostic mycobacteriology to distinguish *M. tuberculosis* from animal-adapted members of the MTBC. *M. tuberculosis* possesses high levels of nitrate reductase activity relative to MTBC isolates that rarely infect humans, which corresponds to promoter mutations upstream of the nitrate reductase operon in these isolates [144]. A recent gene expression study comparing the five distinct MTBC lineages found that nitrate reductase is highly expressed in lineages L2 and L4 but is repressed in lineages L1 and L6 during infection in macrophages [92]. This finding is consistent with previous observations that clinical isolates from lineage L1 are variable with respect to their nitrate reductase phenotype [145]. In *M. tuberculosis*, nitrate reductase activity has been associated with mitigating acid and reactive nitrogen stresses under hypoxic conditions [146], however it is unclear if nitrate reductase activity confers a specific fitness advantage in different MTBC lineages. The mycobacterial nitrate reductase likely requires molybdenum cofactor (MoCo) for enzymatic activity [147,148]. Interestingly, MTBC isolates in clade A3 lack many genes thought be involved in MoCo biosynthesis (see below), suggesting that a specific patho-adaptation has occurred in this clade that may further reduce nitrate reductase activity in isolates from clade A3.

11. Metals and Metabolism in *M. tuberculosis*

M. tuberculosis requires metals such as iron and copper for growth, and these elements are linked to growth and pathogenicity of *M. tuberculosis* in macrophages [149]. Iron is required for the activity of numerous enzymes, as it is either attached in a heme nucleus or coordinated by amino acid side chains within enzyme active sites. During infection in macrophages *M. tuberculosis* induces the expression of many genes associated with acquiring and sequestering iron [74,75,150]. *M. tuberculosis* acquires iron via siderophore scavenging from iron-loaded transferrin in the endocytic pathway [51,53] or by importing heme [151]. Metal composition analysis by X-ray fluorescence in infected macrophages found that *M. tuberculosis* containing phagosomes have high concentrations of iron, a property that was dependent on the production of siderophores and did not occur in nonpathogenic mycobacteria [152]. Similarly, copper plays an important role in *M. tuberculosis* metabolism because it forms the nuclear center of the aa_3-type cytochrome *c* oxidase [141,153]. This cytochrome oxidase is one of two enzymes in *M. tuberculosis* that are known to require copper for activity and this cytochrome oxidase is critical for oxidative respiration. Copper also accumulates in the phagosomes of infected macrophages, which presumably provides the bacterium necessary levels of this metal [152]. While it is not clear how copper is imported by *M. tuberculosis*, it appears that excess copper is associated with adverse effects on *M. tuberculosis*, and the bacterium has several ways to remove or inactivate this metal to avoid toxicity (see below).

12. Molybdenum Cofactor in *M. tuberculosis*

MoCo is a cyclic redox cofactor required by a subset of enzymes to catalyze carbon, nitrogen, and sulfur metabolic reactions across all kingdoms of life [154]. The *M. tuberculosis* genome encodes numerous putative MoCo requiring enzymes, including the nitrate reductase NarGHJI [155] along with an expanded set of genes thought to be involved in MoCo biosynthesis [156]. Although the role of MoCo in *M. tuberculosis* metabolism during infection remains unknown, the expansion of genes involved in MoCo is a unique feature of MTBC pathogens [157]. The biosynthesis of MoCo in *M. tuberculosis* is a complicated and poorly-understood process that likely involves multiple redundant enzymes [156]. A subset of these MoCo biosynthetic genes (*moaA1-D1*) was likely acquired by *M. tuberculosis* during an ancient horizontal gene transfer event [158–160] and these genes enhance survival of *M. tuberculosis*

during hypoxia [147]. Interestingly, *M. orygis* isolates in the clade A3 carry a unique 13 gene deletion mutation (RD12oryx) that disables multiple putative MoCo biosynthetic genes and a transcriptional regulator of the *moaA1-D1* operon [147,161]. It is unknown if these bacteria produce MoCo but, it is tempting to speculate that a unique patho-adaptation has occurred in these isolates that impacts the ability of these bacteria to produce MoCo and tolerate hypoxia.

13. Immunological Pressures on *M. tuberculosis* Metabolism

Macrophages are not a static niche that simply supplies nutrients for *M. tuberculosis* and immunologic pressures imposed by macrophages modulate the bacterium's ability to engage metabolic pathways and utilize host-derived nutrients. Immune activation induces expression of the macrophage immunoresponsive gene-1 (Irg1 or aconitate decarboxylase 1) [162]. This enzyme disables the macrophage mitochondrial TCA cycle by decarboxylating cis-aconitate [162] leading to the accumulation of acetyl-CoA, which can be diverted into fatty acid synthesis by the host cell. Additionally, the cis-aconitate decarboxylation reaction also produces itaconic acid and this metabolite inhibits both *M. tuberculosis* and macrophage metabolism. Itaconic acid inhibits Icl1 in *M. tuberculosis*, which limits the bacterium's ability to utilize intermediates from cholesterol and fatty acid degradation [109]. Itaconic acid also regulates mitochondrial respiration by inhibiting succinate dehydrogenase, leading to the production of excess succinate that drives glycolytic metabolism in the macrophage and modulation of the immune response [163]. Amino acids are also a heavily contested nutrient during infection. IFN-γ-induced activation of macrophages increases expression of the enzyme indoleamine 2, 3-dioxygenase 1 (Ido1) [164]. The host protein Ido1 degrades tryptophan and depletes levels of this critical amino acid in activated cells; however, *M. tuberculosis* compensates for this by synthesizing its own tryptophan [165,166]. Similarly, mice infected with *M. bovis* increases overexpression of Ido1 although this bacterium also synthesizes its own tryptophan [167]. Given that activation of Ido-1 is produced in response to the cell mediated adaptive immunity it is expected that all members of the MTBC will experience this constraint and rely on the synthesis of this amino acid to replicate during infection.

Macrophages also possess an armamentarium of metal-based mechanisms to eliminate intracellular pathogens [168]. Although copper and zinc are important metals in *M. tuberculosis* physiology these metals also constitute an important cellular defense when these ions accumulate within macrophage lysosomes during infection [168]. The P-type ATPase ATP7A mediates phagosomal accumulation of copper, which functions as a defense mechanism to intoxicate bacterial pathogens [169]. To mitigate copper overload and toxicity *M. tuberculosis* either actively secretes copper [170,171], or sequesters the metal in an inert, protein-bound form [172]. Macrophages are thought to deliver zinc into phagosomes through the activity of one or more transporters [173] and *M. tuberculosis* mitigates zinc poisoning by actively exporting the metal [174]. The understanding that *M. tuberculosis* is equipped to mitigate copper and zinc toxicity during infection in macrophages is a relative recent finding. It will be exciting to determine how other MTBC members adapt to these metals during infection.

14. Acidic pH and PhoPR

M. tuberculosis senses and adapts to the environmental conditions within macrophage phagosomes [48] and one major signal sensed by *M. tuberculosis* is fluctuating acidity [150]. *M. tuberculosis* senses and adapts to low pH environments via the two-component regulator system, PhoPR [175]. The PhoPR system is critical for virulence/pathogenesis that controls the expression of genes in a large virulence regulon involved in metabolism [176], virulence protein secretion (Esx) [177,178], and the biosynthesis of methyl-branched lipids (acylated trehalose, SL, PDIM) [175,179].

Most human-adapted isolates of the MTBC have a functional PhoPR system while *M. africanum* and MTBC isolates that rarely infect humans have a mutation in the sensor domain of the PhoR kinase (PhoR G71I), which disables this protein [180]. It is well established that many genes in the PhoPR regulon are required for virulence/pathogenesis. Bacteria that carry a non-functional PhoR also have

compensatory mutations that partly restore expression of the PhoPR regulon [181]. Specifically, some lineages with a non-functional PhoR have a secondary deletion termed RD8. The RD8 mutation restores the expression and secretion of virulence proteins (Esx) in *M. africanum* lineage L6 and related isolates [181]. Isolates in *M. africanum* lineage L5 also carry a non-functional PhoR but these bacteria do not have a RD8 mutation. *M. africanum* lineage L5 bacteria are still able to express and secrete virulence proteins (Esx) indicating that an unidentified mechanism compensates for the PhoR mutation in this lineage [182].

Methyl-branched lipid biosynthesis is also regulated by the PhoPR system and these lipids have long been associated with virulence in *M. tuberculosis* [183]. Additionally, synthesis of these lipids can mitigate metabolic toxicity associated with cholesterol and fatty acid metabolism (see above) [56]. It is tempting to speculate that low pH-dependent activation of methyl-branched lipid synthesis via PhoPR activation could couple host-derived lipid utilization pathways in *M. tuberculosis* to promote pathogenesis in macrophages.

These methyl-branched polyketide lipids may also play a role in transmission. Specifically, *M. bovis* isolates rarely transmit between humans, with a notable exception being the *M. bovis* isolate strain B. This strain of *M. bovis* efficiently transmits between humans and has been associated with high mortality among HIV-infected persons [184]. Molecular characterization of this strain revealed an IS*6110* insertion upstream of *phoP* that enhances transcription of *phoP* [185]. Importantly, when this mutation was engineered into a laboratory isolate of *M. bovis* this mutation allowed the bacteria to produce SL, a methyl-branched polyketide lipid [180]. This observation suggests that the virulence and/or host range of MTBC bacteria can be altered by the PhoPR-dependent production of methyl-branched polyketide lipids such as SL.

15. Concluding Remarks

Differences clearly exist between the MTBC members with respect to pathogenesis, host range, and transmission there are also similarities with these pathogens. This review focused on the specialized metabolism that allows *M. tuberculosis* to survive in macrophages because this is a common survival strategy used by MTBC pathogens that rarely infects humans. Comparative genomic approaches across members of the MTBC revealed several key differences regarding virulence and immunogenicity. Similarly, we are optimistic that comparative approaches focused on metabolism will enhance our understanding of this important group of pathogens and the animals they infect.

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