Multiomics Integration of Tuberculosis Pathogenesis

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Jae Jin Lee, Philip Sell, and Hyungjin Eoh

Unity, not uniformity, must be our aim. We attain unity only through variety. Differences must be integrated, not annihilated, not absorbed.

Mary Parker Follett

Summary

Advances in high-throughput technology have made it possible to quantitatively monitor changes in multiple sets of biological molecules under different environmental stresses. Microbial adaptation to stresses can be monitored by genomics, transcriptomics, proteomics, and metabolomics. When combined, the resulting multiomics approach provides a much more comprehensive perspective of biological systems than using any single omics alone. Integrated multiomics has improved our understanding of the complex adaptive mechanisms of pathogens and allows for more accurate predictions of pathogenic outcomes. A vast amount of research has been carried out on tuberculosis (TB)

J. J. Lee · P. Sell · H. Eoh (✉)
Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA
e-mail: heoh@usc.edu

J. J. Lee
e-mail: jaejinle@usc.edu

P. Sell
e-mail: psell@usc.edu

J. J. Lee · P. Sell · H. Eoh
Integrated Science Association (ISA), Universal Scientific Education and Research Network (USERN), Los Angeles, CA 90033, USA

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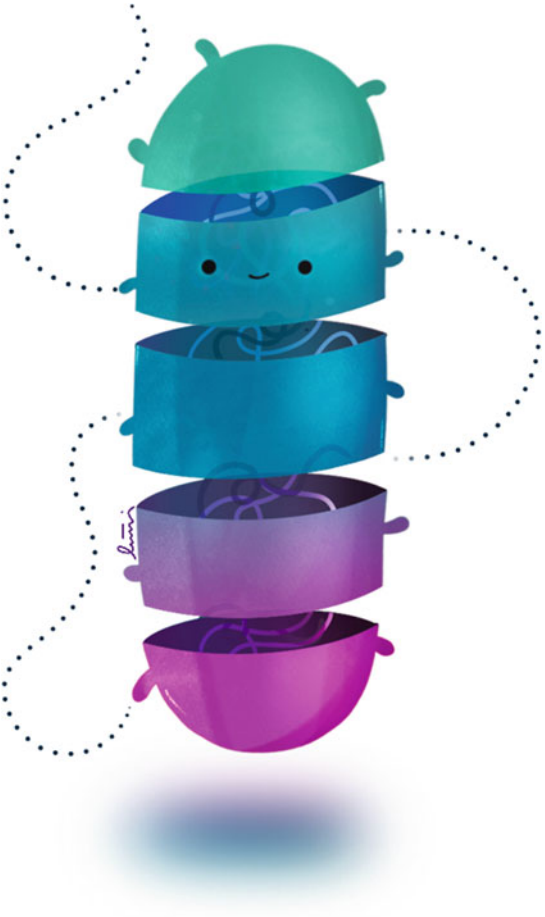
pathogenesis. These studies decipher the biological molecules, pathways, and components of *Mycobacterium tuberculosis (M. tb)*, the etiological agent of TB, involved in the adaptive strategies required for virulence. This chapter summarizes our current knowledge drawn from studies investigating the metabolic adaptation of *M. tb* and its survival in different phenotypic states. The collective interpretation of diverse but essential metabolic networks in *M. tb* will provide new insights for more effective TB interventions.

Graphical Abstract



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Keywords

Metabolic essentiality · Metabolic remodelling · Multiomics · Pathogenesis · Phenotypic heterogeneity

1 Introduction

Tuberculosis (TB) research continues to persevere towards developing efficient treatment. Despite continuous advances in research technology to reveal TB pathogenesis, *Mycobacterium tuberculosis (M. tb)* remains a formidable foe against the countless therapies that have been invented to date [1–3].

The hallmark of *M. tb* pathogenesis is its ability to adapt to various antimicrobial environments identified within hosts. *M. tb* can replicate even under an intact immune system; soon after recruitment, functional immune cells release reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), then in response, *M. tb* slows its replication rate, which enhances the phenotypic heterogeneity within the population [4–12]. Adequate immune responses recruit various immune cells, which convene into a multicellular structure called a granuloma which functions as a trap to keep uncleared *M. tb* bacilli within a restricted area [13, 14]. The interior of the granuloma is full of biochemical stresses, including hypoxia, nutrient starvation, low pH, and accumulation of ROS and RNI, wherein the majority of *M. tb* bacilli resides in the non- or slowly- replicating state by limiting its replication [15–20]. Thus, granuloma formation is a critical innate immune strategy [21, 22]. As a countermeasure, inside the granuloma, *M. tb* survives by shifting to a non-replicating persistent (NRP) state. Infected individuals with properly functioning immune systems may present clinically asymptomatic where *M. tb* mostly lives in an NRP but a metabolically active state. The NRP state of *M. tb* is phenotypically tolerant to nearly all TB antibiotics because they target cellular processes active only during replication [23–27]. This phenotypic quiescence and drug tolerance explain long TB treatment durations, which provides favorable conditions for the emergence of drug-resistant (DR) mutations [28–36]. Moreover, this NRP population serves as a reservoir for disease reactivation [37, 38]. A study at Cornell University using an NRP-TB mouse model showed that *M. tb* bacterial burden was undetectable after bactericidal antibiotic treatment, but reactivation occurred in response to immunosuppressive agents, such as glucocorticoids, in the absence of new infections [39–41].

Although immunological and biochemical stresses slow its replication, a significant portion of *M. tb* continues to replicate throughout infection in mouse models [9, 42]. This implies that the bacillary number in the NRP state is largely maintained by balancing the number of replicating, non-replicating, and dying bacilli at the site of infection under the active immune system in a state of phenotypic heterogeneity. Within granulomas, *M. tb* is trapped inside macrophages or

in extracellular niches at necrotic centers of granulomas. *M. tb* can also be found in the upper lobe of the lung on the inner cavity epithelium, where they can exit the NRP state [43–46]. Thus, the physiological understanding of *M. tb* bacilli in various phenotypic states is the unmet clinical and scientific need to develop more powerful therapeutic interventions.

As a unicellular organism, diversity is key for *M. tb* to survive harsh conditions [47, 48]. Diversity in a microbial population has been considered an important strategy to maximize adaptation in rapidly changing environments through metabolic flexibility and mutual interaction with neighboring siblings. *M. tb* always retains a level of metabolic diversity; subpopulations that encode identical genetic information can exhibit phenotypic heterogeneity [9–11, 49, 50]. This strategy allows *M. tb* to secure its species as a bet-hedging tactic [51–53]. This heterogenous population is typically composed of a random mixture of phenotypic states from fully replicating to completely NRP [53, 54]. Consequently, *M. tb* relies on nongenetic mechanistic strategies, including optimization of cellular fitness and metabolic shifts, which are difficult to elucidate by analyzing a single set of biological molecules.

Whole-genome sequencing was first conducted to uncover the *Haemophilus influenzae* genome in the mid-1990s [55, 56]. This served as a milestone in systems biology, but the volume of data went beyond a complete interpretation at the time. Bioinformatic tools have been co-developed to understand relevant genetic information among copious data. Details on transcription of genetic information to mRNA, subsequent translation into proteins, and finally, the substrates and products of protein complexes all must be included to reach a more comprehensive representation of the data. The nuanced intermediates involved in metabolic networks are all crucial to holistically understanding microbial pathogenesis [57–59].

This chapter provides the characteristics associated with phenotypic heterogeneity of *M. tb* that have been validated by the multiomics approach. We focus on the metabolic activities essential for survival in each major phenotypic state: replicating, NRP, and reactivation. All cellular processes stem from the foundation of metabolism, thereby making metabolism an explication of pathogenicity. This perspective will direct efforts in a more sophisticated way to better develop effective treatments and strategies to address the *M. tb* pandemic and quell its widespread transmission.

2 Multiomics Integration to Study *M. Tuberculosis* Pathogenesis

2.1 Multiomics Technology as a Window to Visualize *M. Tuberculosis* Physiology

The whole-genome sequence of *M. tb* H_{37}Rv was first published in 1998 [60]. This genomics study identified over 3900 open reading frames, of which less than 50% were annotated with known functions. Manipulating gene function using gene

knockouts aided in defining the essential pathways for *M. tb* growth and adaption to adverse environments [61, 62]. Single-gene knockout methods have been used alongside global methods, such as transposon (Tn) mutagenesis, to inactivate genes. Tn libraries were generated by random insertion within the *M. tb* genome and monitored for essentiality in the presence of selective conditions. DNA microarrays were then used to map Tn abundance, termed Tn site hybridization (TraSH). Sassetti et al. applied the TraSH method using a bacteriophage vehicle and successfully identified 614 genes essential for in vitro growth [63, 64]. These genes are involved in biosynthesizing amino acids, cofactors, and nucleic acids, including many unknown functions.

Conditional expression methods use an inducible promotor (TetON or TetOFF) to investigate genes' essentiality and functions. This strategy was used by Schnappinger et al. to control 474 genes by Tet regulation which identified greater than 8.5 million chemical-genetic interactions [65–67].

More recently, Clustered Regularly Interspaced Short Palindromic Repeats Interference (CRISPR*i*) was applied to essential gene screening in mycobacterial genomics [68–70]. This system works by targeting dCas9 nuclease, with nonfunctional nuclease activity, to *M. tb* genes with a sgRNA (single guide RNA). When the dCas9-sgRNA complex is formed, transcription of targeted genes is stalled by sterically blocking access of RNA polymerase to the promotor. The level of gene silencing is determined by sgRNA length and sequence, which makes it possible to study the impact of essential gene depletion where traditional approaches to completely knock out genes would be lethal to *M. tb*. The CRISPR*i*-dCas9 system was also used to generate an approximately 90,000 sgRNA library, enabling high-throughput platforms to screen for essential genes [68]. Rock et al. used sgRNA to make *M. smegmatis* folate metabolism hypomorphs to show the essentiality of this pathway for replication [69]. CRISPR*i* is a useful tool for genetic studies, but a potential caveat is the off-target effects from nonspecific interactions of dCas9.

Studying the *M. tb* transcriptome adds another avenue to connect genomic information [71]. Transcriptomics can take snapshots of gene expression levels in *M. tb* during adaptation to various environments, making it an excellent method to explore *M. tb* pathogenesis. Beyond understanding *M. tb* physiology, transcriptomics has been useful in assigning gene function and essentiality, discovering drug targets, and exploring an antimicrobial's mode of action [72–75]. Transcriptomics has identified the intermediary pathways required by intracellular *M. tb* to utilize host lipids: the β-oxidation pathway, glyoxylate shunt, methylcitrate cycle (MCC), and cholesterol metabolism [65, 76–81]. Another transcriptomics study found induction of glutamate synthase (GltB) and glutamate decarboxylase (GadB) as an adaptive strategy to counteract intracellular acidification arising from MCC activation [82]. Transcriptomics profiling of in vitro NRP models revealed significant remodeling in electron transport chain (ETC) activities [83, 84]. Combining transcriptomics with gene essentiality datasets offers a beginning to the multiomics understanding of TB pathogenesis.

Metabolomics is the holistic evaluation of metabolite networks in an organism or system of biological study. It is crucial for understanding the metabolic processes underlying *M. tb* adaptation to adverse environments [57, 85–90]. Carvalho et al. aerobically grew *M. tb* in the presence of fully ^{13}C labeled glycerol, acetate, or glucose and analyzed the ^{13}C enrichment of each metabolite in central carbon metabolism (CCM) pathways [91]. They demonstrated that *M. tb* could co-catabolize multiple carbon substrates simultaneously through glycolysis and the tricarboxylic acid (TCA) cycle. This co-catabolism is mediated by the compartmentalization of each carbon component to a distinct metabolic direction [92]. A study led by Agapova et al. observed that *M. tb* could also utilize multiple amino acids as nitrogen sources [93]. Serafini et al. found a new role of MCC as a source of propionyl-CoA and cell wall lipids through the reverse direction by assimilation of pyruvate and lactate [94]. Additionally, Dutta et al. used metabolomics to compare wild-type and Rel-deficient *M. tb* to verify that the Rel stringent response regulator functions in *M. tb's* transition to the non-replicating, quiescent NRP state [95]. As such, metabolomics defines metabolic networks and helps pinpoint the essential pathways used in the adaptive strategies of *M. tb*.

2.2 Metabolic Networks of *M. Tuberculosis* in Diverse Phenotypic States

2.2.1 Actively Replicating State

Permissive Carbon Sources

Intracellular pathogens acquire nutrients needed to generate energy and biomass from the host [96]. It is technically challenging to study intracellular *M. tb* physiology due to the indirect nature of all experimental trials; earlier methods to probe main carbon sources largely relied on infection with auxotrophs [97]. Zimmerman et al. integrated information from metabolomics and transcriptomics data to reveal that intracellular *M. tb* utilizes up to 33 different nutrients from the host, of which three are solely used for biomass and the remaining 30 for ATP biosynthesis [98]. These included various subclasses of lipids such as monoacylglycerols and phosphatidylinositol phosphate, suggesting that *M. tb* is exposed to various nutrients within macrophages.

The foregoing multiomics integration of intracellular *M. tb* datasets pinpointed cholesterol as one of the most prevalent permissive carbon sources by detecting 4,5-9,10-diseco-3-hydroxy-5,9,17-tri-oxoandrosta-1(10),2-diene-4-oic acid (DSHA) accumulation. Since DSHA is a non-mammalian metabolite, it is degraded from cholesterol by *M. tb*. Supporting this, during infection, they observed host cholesterol levels decrease by $\sim$ 80% and depletion of other cholesterol derivatives [99].

Simultaneous consumption of multiple carbon and nitrogen sources illustrates that *M. tb* has evolved highly modular metabolic networks that adapt to nutritionally adverse environments. Genomics studies have demonstrated that *M. tb* lacks the classical phosphotransferase (PTS) and carbon catabolite repression

(CCR) system–canonical adaptive strategies used to consume external carbon nutrients, especially by gram-negative and gram-positive bacteria [60, 91]. Hence, the metabolic network topology of *M. tb* is evolutionarily designed for optimal and unique pathogenesis.

Cholesterols

Although *M. tb* can simultaneously co-catabolize multiple carbon and nitrogen sources, accumulating genomics studies designate cholesterol as the most probable carbon source for *M. tb* growth and even survival in the NRP state from various in vitro and in vivo models [100–102].

Mce (mammalian cell entry) membrane proteins (Mce1-4) import lipid substances into *M. tb* (Fig. 1). Pandey et al. identified Mce4 as the major import route of cholesterol [103]. Mce4 is thought to confer *M. tb* pathogenesis by promoting its intracellular growth, whereby *mce4* deficient *M. tb* replicates poorly in media with cholesterol as a sole carbon substrate and in mice [104]. Multiple transcriptomics and proteomics analyses showed significantly higher expression of *mce1–mce4* transcripts and their protein levels in later stages of growth than in the early exponential stage, indicating growth phase-dependent functionality of Mce proteins. The functional essentiality of Mce4 was supported by the enhanced expression level of *mce4* in *M. tb* while residing within macrophages [105].

Once imported, *M. tb* degrades cholesterol to exclusively biosynthesize pyruvate, acetyl-CoA, propionyl-CoA, and succinyl-CoA, all precursor substrates of CCM pathways (Fig. 1) [96]. Combining metabolomics and transcriptome analysis by RNA-Seq from *M. tb* during infection determined overlapping metabolic networks, enzymes, and metabolites, several of which belonged to cholesterol degradation pathways [98, 106]. The final products of cholesterol degradation are now acknowledged as key metabolic intermediates required to fuel *M. tb* CCM, including the TCA cycle, glyoxylate shunt, MCC, methylmalonyl-CoA pathway, and gluconeogenic pathway (Fig. 1) [107, 108]. Propionyl-CoA is an initial substrate for the methylmalonyl-CoA pathway and MCC [109]. Biosynthesis of methyl-branched polyketide lipids and long-chain fatty acids occurs in the methylmalonyl-CoA pathway. The methylmalonyl-CoA and MCC pathways fuel CCM intermediates, thereby balancing toxic effects associated with cholesterol consumption, propionyl-CoA accumulation, and biosynthesis of essential intermediary metabolites of the TCA cycle [81, 109, 110]. Indeed, *M. tb* bacilli deficient in isocitrate lyase (ICL), the last enzyme in MCC, stopped replication and died from an accumulation of toxic intermediates and starvation of essential TCA cycle intermediates [80, 81]. Isotope metabolomics of *M. tb* deficient in ICL (Δ ICL) proved this vulnerability by showing that metabolic defects in Δ ICL stemmed from the conversion of the MCC into a dead-end pathway. Subsequent accumulation of MCC intermediates, such as 2-methylcitrate and 2-methylisocitrate, led to altered NADH/NAD + ratio, membrane potential, and intracellular acidification [81].

Vitamin B12-dependent shunting of propionyl-CoA through the methylmalonyl-CoA pathway minimizes the MCC intermediate-mediated toxicity while biosynthesizing lipids including sulfolipid-1 (SL-1) and phthiocerol

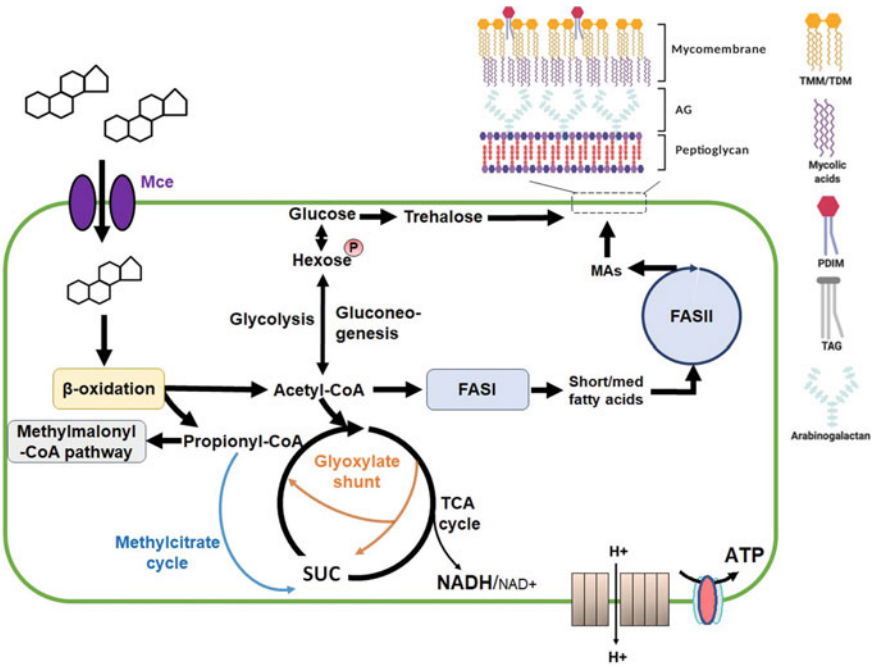

Fig. 1 Metabolic networks of replicating *M. tb*. *M. tb* membrane protein, Mce 4 (Mammalian Cell Entry), is used to import cholesterol. Cholesterol serves as a substrate of β-oxidation that leads to the biosynthesis of acetyl-CoA, propionyl-CoA, and pyruvate. These metabolites are substrates of the TCA cycle, glyoxylate shunt, methylcitrate cycle, methylmalonyl-CoA pathway, and gluconeogenesis. Acetyl-CoA is a substrate of the TCA cycle and subsequent production of NADH, a reduced electron carrier required for the production of ATP via electron transport chain (ETC). The set of FASI and FASII activities is required for the biosynthesis of mycolic acids (MA). Mycolic acids are condensed with trehalose to produce trehalose dimycolate (TDM), one of the most prevalent PAMPs (pathogen-associated molecular pattern). Hexose P, glucose phosphate and its isomers; MAs, mycolic acids; TMM, trehalose monomycolate; AG, arabinogalactan; SUC, succinate; TAG, triacylglycerol; PDIM, phthiocerol dimycocerosate. (*Created with BioRender.com*)

dimycocerosate (PDIM) [81, 82, 109]. *M. tb* enhances the biosynthesis of PDIM and SL-1 when residing inside the host by coupling to catabolic cholesterol metabolism. This metabolic channeling is associated with *M. tb* pathogenesis because PDIM is known to aid in the recruitment and invasion of macrophages at the site of infection and help evade immune responses by masking cell surface PAMP (pathogen-associated molecular pattern) molecules [111, 112].

Lee et al. performed metabolomics analysis with BCG (Bacillus Calmette–Guérin), a live TB vaccine strain, and *M. tb* H_{37}Rv after culturing in media containing propionate or inside the macrophages [82]. The results supported an essential role of MCC in optimal intracellular replication and *M. tb* pathogenesis. They also observed an increase in glutamate synthase (GltB/D) activity, converting

glutamine to glutamate and GABA (γ-aminobutyric acid), as a metabolic effort to neutralize MCC intermediates and propionyl-CoA mediated toxicity. Indeed, increased GltB/D activity improved the survival of Δ ICL cultured in media containing propionate. Metabolic networks in *M. tb* are interconnected such that MCC intermediates or propionyl-CoA mediated toxicity on ETC and bioenergetics can be corrected by GltB/D activity in nitrogen metabolism, an example of the process termed metabolic adaptation. Collectively, the metabolic plasticity of *M. tb* required for permissive carbon uptake and consumption is thus accepted as a hallmark of its pathogenesis.

Antibiotic Targets of Actively Replicating *M. Tuberculosis*

Mycolic Acid Synthesis

Isonicotinic acid hydrazide, or isoniazid (INH), has been studied extensively and is a main component of the conventional first-line TB drug regimen. INH is significantly more potent in killing replicating *M. tb* than all other TB antibiotics [113–116]. The antimicrobial kinetics of INH was delayed by around four days, during which accumulated INH could not be washed from INH-susceptible *M. tb* while it could be washed away from resistant bacilli [116]. The accumulation of INH within INH-susceptible strains was accompanied by a wrinkled cell surface phenotype and a loss of its acid-fastness, a bactericidal phenotype specifically shown after INH treatment. Jacobs et al. characterized *M. smegmatis* mutants resistant to INH to find the drug target to be InhA, an NADH-dependent enoyl-ACP reductase [117–119]. InhA is part of the type II fatty acid synthesis pathway (FAS II) for mycolic acid biosynthesis (Fig. 1). The missense mutations found in these mutants at inhA and *katG* (catalase-peroxidase) conferred an order of magnitude increase of INH minimal inhibitory concentration (MIC). They could be rescued by replacing wild-type *inhA* through allelic exchange [120]. Inversely, the degree of INH resistance can be predicted by identifying the mutations of *inhA*, although missense mutations at other genes, including *kasA* or *katG,* must also be considered. A study confirmed that INH specifically inhibits the InhA step in the FAS II pathway by two-dimensional clustering of whole protein expression profiles of *M. tb* after INH treatment. Wilson et al. used a microarray hybridization to confirm that the mode of action of INH is associated with FAS II by showing operonic clustering of the genes encoding FAS II (Rv2243–Rv2247) and FbpC (Ag85C) as interacting partners.

INH effects on *M. tb* mycolic acid biosynthesis and its specificity to actively replicating *M. tb* suggest that the metabolic activities linked to mycolic acid biosynthesis are essential for *M. tb* replication and survival.

Membrane Energetics

Energy production from the ETC occurs via oxidative phosphorylation (OXPHOS), a ubiquitous and essential metabolic pathway for *M. tb* replication [121, 122]. Nutrients are used to fuel OXPHOS and subsequently generate an electrochemical gradient, called the proton motive force (PMF), that serves as a major source of

intracellular protons and ATP by driving ATP synthases (Fig. 1). In *M. tb*, the generation of the PMF is mediated by the proton-pumping components of ETC, including cytochrome bc1-aa3 complex and, albeit a less exergonic reaction, cytochrome bd oxidase [84, 123, 124]. Recently, the discovery of small molecules targeting proton-pumping components in *M. tb* ETC has targeted the cytochrome bc1 subunit [125–127]. The lead compounds to date are a series of imidazopyridine amides. The most promising drug candidate of the series, Q203, is currently in phase II clinical trials under the FDA Investigational New Drug Application [128].

The discovery of bedaquiline (BDQ), a mycobacterial F1F0-ATP synthase inhibitor, validated OXPHOS as an essential component for survival in the replicating state [129–131]. BDQ became an important TB drug to treat multidrug-drug-resistant (MDR) or extensively drug-resistant (XDR) TB patients. BDQ showed an unusually slowed time-dependent killing termed as weak-early bactericidal activity, where the four $\log_{10}$ reductions in bactericidal phase just began four days after treatment following a period of bacteriostatic activity only [129, 132–135]. Transcriptomics and proteomics analysis of *M. tb* treated with BDQ observed induction of around 39 genes that belong to the dormancy regulon [135]; these genes may function to counteract damaged F1F0-ATP synthase, which would explain the temporal maintenance of bacteriostatic viability during initial exposure. Recent genetics studies using *Staphylococcus aureus* and *Escherichia coli* showed that ATP depletion is a signal that triggers bacterial dormancy and persistence [136–140]. The early bactericidal activity of BDQ differs depending upon the carbon substrates available in the media. Fermentable energy sources can be consumed for ATP biosynthesis through glycolysis or OXPHOS. However, *M. tb* cannot break down fatty acids by glycolysis, so in this case, ATP production solely relies on OXPHOS. *M. tb* displayed increased susceptibility to BDQ when cultured in media containing fatty acids as the only source of carbon [135]. A recent metabolomics study led by Wang et al. validated BDQ's effect on *M. tb* in a replicating state with the combination of direct ATP biosynthesis inhibition via interfering with F1F0-ATP synthase activity and indirect metabolic consequences arising from hundreds of annotated ATP-dependent reactions including glutamine synthase activity [141].

2.2.2 Non-Replicating Persistent State

Alternate Carbon Sources

NRP *M. tb* downregulates activities of key components in the ETC, which decreases OXPHOS activity and ATP biosynthesis to approximately 10% to that of its replicating counterparts [83, 142, 143]. The notable characteristic of NRP *M. tb* is its phenotypic drug tolerance or nonheritable resistance, which allows persistence under effective chemotherapies [31, 144–147]. This is largely because antibiotics that effectively kill replicating *M. tb* are no longer effective in killing NRP *M. tb* (Tables 1 and 2). For this reason, the physiology of NRP *M. tb* has emerged as a central feature of its pathogenesis [26]. Sarathy et al. used an established in vitro nutrient starvation NRP model to measure the intracellular level of diverse anti-TB

Table 1 Antibiotics targeting actively replicating *M. tb*

Sites of action	Antibiotics	Mechanisms of action
Cell wall	Isoniazid	Inhibits nicotinamide adenine dinucleotide (NADH)-specific enoyl-acyl carrier protein (ACP) reductase involved in the fatty acid synthesis
	Ethambutol	Inhibits arabinosyl transferases involved in cell wall biosynthesis
	D-Cycloserine	Inhibits synthesis of peptidoglycan and cell wall maintenance
	Ethionamide	Inhibits the *inhA* gene product enoyl-ACP reductase
	Prothionamide	
	SQ109	Inhibits the MmpL3 TMM exporter
DNA replication	Moxifloxacin	Inhibits the ATP-dependent enzymes topoisomerase II (DNA gyrase) and topoisomerase IV
	Gatifloxacin	
	Levofloxacin	Inhibits the DNA-gyrase, which in turn inhibits the relaxation of supercoiled DNA
Transcription	Rifampin	Inhibits the activity of the essential *rpoB* gene product β-subunit of DNA-dependent RNA polymerase
	Rifabutin	
	Rifalazil	
	Rifapentine	
Translation	Amikacin	Inhibits protein synthesis by binding to the conserved A site of 16S rRNA in the 30S ribosomal subunit
	Kanamycin	
	Streptomycin	
	Capreomycin	Inhibits protein synthesis by interacting with the ribosome
	Clarithromycin	Inhibits protein synthesis by binding to the 50S ribosomal subunit
	Linezolid	Inhibits protein synthesis by binding to 23S rRNA in the early phase to prevent proper binding of formyl-methionine tRNA
Cytoplasmic process	Para-aminosalicylic acid (PAS)	Acts as a metabolic precursor (prodrug) that generates a toxic dihydrofolate analog that subsequently inhibits DHFR (dihydrofolate reductase) activity
ATP synthesis	Bedaquiline	Inhibits mycobacterial F1F0-ATP synthase by binding to proton pump subunit

antibiotics such as fluoroquinolones, rifampin, and linezolid; they found that penetration in NRP state bacilli was significantly lower than in replicating bacilli [148–150]. Efflux pump inhibitors were used to confirm that reduced accumulation of antibiotics within the bacilli was independent of efflux processes [151]. Recent ^{13}C isotope metabolomics studies using the in vitro biofilm culture system also showed that the relationship between the enrichment of NRP populations and the ATP biosynthesis and consumption rate of external carbon sources is inversely

Table 2 Antibiotics targeting non-replicating persistent (NRP) *M. tb*

Antibiotics	Mechanisms of action
Bedaquiline	Inhibits mycobacterial F1F0-ATP synthase by binding to proton pump subunit
Pretomanid (PA-824)	Generates the ROS
AM-0016	Causes cell envelope damage and rapidly collapses membrane potential (A novel xanthone-based antibacterial)
Pyrazinamide	Pyrazinamide enters bacteria and is converted into POA. Under acidic pH, POA accumulates inside the cells and causes a deenergized membrane and acidification of the cytoplasm
Rhodanine (D155931)	Targets dihydrolipoamide acyltransferase (DlaT) activity
TCA1	TCA1 is activated by decaprenyl-phosphoryl-β-D-ribofuranose oxidoreductase DprE1 and MoeW, enzymes involved in the cell wall and molybdenum cofactor biosynthesis

proportional [34]. Similar findings were observed in otherwise permissive *M. tb* adapting to hypoxic conditions [142, 143], suggesting that NRP *M. tb* can withstand adverse environmental stresses by depleting its ATP level to restrict the uptake of external antibiotics and carbon sources.

Existing knowledge of *M. tb* in an NRP state derives from experimental models of varying physiologic relevance and, despite controversial debates, almost all evidence includes alteration in ETC activity. Even NRP *M. tb* needs a basal metabolic activity level sufficient to maintain an energized membrane potential, PMF, and core cellular processes [83, 142, 152]. For example, when biosynthesizing low levels of ATP, NRP *M. tb* must continuously recycle the reducing equivalent, NAD^+, to maintain the PMF. A study using isotopologue analysis and metabolomics examined the change of CCM pathway intermediates during transitioning into NRP state [142, 152], confirmed by a significant change in the NADH/NAD^+ ratio. As a compensatory mechanism, NRP *M. tb* initiates a fermentative respiratory strategy by net incorporation of CO_2 and active secretion of succinate by activating a reversed TCA cycle (Fig. 2). Another metabolomics work measured TCA cycle intermediates during the NRP state metabolism shift to show the critical role of succinate. However, fatty acids were provided to mimic conditions relevant to the host. Eoh et al. discovered that the glyoxylate shunt mediates hypoxia adaptation by contributing to the biosynthesis of succinate and glycine as an end product of the glyoxylate shunt, which is divergent from its traditional role in fatty acid metabolism required for active replication (Fig. 2) [142].

Transcriptomics studies using nutrient starvation identified the general depletion of genes associated with aerobic ETC components, suggesting metabolic changes that are similar to those during hypoxic adaptation [153–155]. These include upregulation of ICL and fumarate reductase with downregulation of F1F0-ATP synthase and type I NADH dehydrogenase (Ndh1). Separate transcriptomics and

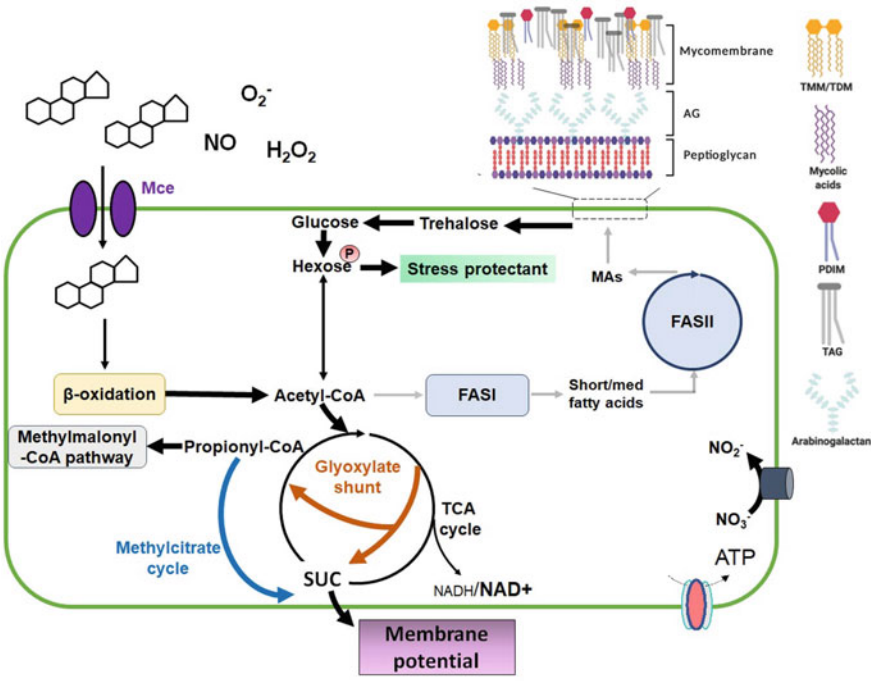

Fig. 2 Metabolic remodeling occurring within NRP *M. tb*. When adapting to biochemical stresses, *M. tb* restricts the uptake of external carbon sources, including cholesterol, while depleting its ATP level. Host fatty acids are still used as the primary source of the glyoxylate shunt, methylcitrate cycle, and methylmalonyl-CoA pathway. Methylmalonyl-CoA pathway contributes to host immune evasion through biosynthesis of phthiocerol dimycocerosate (PDIM). Meanwhile, NRP *M. tb* utilizes preexisting trehalose to fuel glycolysis and the pentose phosphate pathway (PPP) as an alternate source for ATP and antioxidants. Shunting trehalose towards the biosynthesis of glycolysis and PPP intermediates results in depletion of TDM content, which allows for the stealth invasion under an intact immune system. Without O_2, nitrate (NO_3) or fumarate can be an alternate electron acceptor for NRP *M. tb*, but none of them could support *M. tb* growth. Extra-biosynthesis of succinate (SUC) is secreted as an end product, which serves as an electrogenic process used to maintain membrane potential and ATP biosynthesis(*Created with BioRender.com*)

proteomics studies of *M. tb* following treatment with INH and linezolid displayed dysregulated pathways, including lipid metabolism, cell wall processes, intermediary metabolism, and ETC activity [156–158]. The expression profile of genes in CCM and ETC suggests a significant overlap in the adaptive strategies used by NRP *M. tb* in response to nutrient starvation, hypoxic environment, or even antibiotic treatment [156, 159]. Multiple studies of chronic phase *M. tb* infection in mice and humans showed altered gene expression in *aceA*, *narK2*, *nuo*, *nadC*, *menA*, *lld2*, and *ppdK*, involved in respiration, and *aceA* and *echA15*, involved in lipid metabolism, that paralleled results found from in vitro hypoxia, nutrient starvation, or antibiotic treatment models. The significant overlap of genes

identified from all of these studies is largely restricted to the ETC, indicating that the ETC is a general sensor of stresses. We speculate that NRP *M. tb* uses ETC activity to sense environmental stresses and then restrict the penetration of toxic metabolites for securing survival through adaptive metabolism. Accordingly, NRP *M. tb* can endure significantly limited support from external nutrients.

Two independent metabolomics studies, using NRP *M. tb* recapitulated under the in vitro biofilm system and hypoxia, validated the contribution of preexisting, intrinsic *M. tb* metabolites as an alternate carbon source [34, 143]. Metabolic profiling specific to NRP *M. tb* and multivariate pathway mapping ranked the trehalose metabolism and glycolysis as the two top pathways. Targeted metabolomics showed the depletion of trehalose with a reciprocal accumulation of glucose phosphate, an initial substrate for intermediates of the pentose phosphate pathway (PPP) and glycolysis. This suggests that preexisting trehalose is an internal source of carbon substrates to fuel PPP and glycolysis while transitioning into NRP *M. tb*. The PPP and glycolysis are replenished by preexisting trehalose that serves as an alternative source of ATP, NADPH, and antioxidants to compensate for ATP depletion and limited external carbon sources (Fig. 2). Genetic experiments to interfere with trehalose carbon consumption confirmed that the lack of catabolic trehalose activity invoked *M. tb* hypersensitivity to INH and BDQ.

The Center for Disease Control and Prevention (CDC) predicted that lengthy TB treatment could be shortened significantly by the addition of antimicrobials that target the NRP subgroup within phenotypically heterogeneous populations of *M. tb* [160]. Known stringencies include nutritional deficiency, ROS, RNI, acidic, hypoxic, and membrane perturbing stresses. Understanding the essential cellular processes during NRP transition in response to the stringent environments will provide insights into the desperately needed improvements to existing TB chemotherapies.

Trehalose

Transcriptomics and proteomics studies commonly indicate trehalose metabolism as one of the essential activities required for survival of NRP *M. tb* [161–163]. Trehalose is a natural, nonreducing glucose disaccharide with an α, α-1,1-glycosidic linkage [α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside] [164]. Soon after its discovery, trehalose has been appreciated as a bioprotectant of stress in many biological systems.

Trehalose in *M. tb* is most widely recognized as a structural component of cell wall glycolipids, like trehalose dimycolate (TDM; cord factor) that also performs essential immunomodulatory functions [34, 165–167]. Another example is SL-1 which has major implications for the pathogenesis and transmission of TB [168, 169]. It was found that trehalose auxotrophs of *M. smegmatis* lose their viability in media lacking free trehalose, suggesting the important role of free trehalose in *M. tb*. Free trehalose has been suspected of having two main functions: stress protection and intracellular carbon storage. *M. tb* likely uses trehalose in both ways. In a replicating state, *M. tb* utilizes trehalose as a carbon source and as a substrate for the biosynthesis of cell envelope glycolipids such as TDM and SL-1. A recent

metabolomics study confirmed that trehalose could also serve as an intracellular storage compound that is internally mobilized during the transition to the NRP state (Fig. 2) [34, 143]. Furthermore, shunting trehalose towards the biosynthesis of CCM intermediates for energy production resulted in depletion of TDM and, presumably, SL-1 content. Indeed, the incubation of NRP *M. tb* cells fixed with paraformaldehyde and mouse bone-marrow-derived macrophages (BMDM) showed the diminished ability of induced pro-inflammatory cytokine secretion by BMDM [143]. This result correlates immunoreactivity with the shift in *M. tb* trehalose metabolism during NRP state survival.

Antibiotic Targets of Non-Replicating *M. Tuberculosis*

Altered Membrane Bioenergetics

The essentialities of bioenergetics and the ETC have been proven in studies with NRP *M. tb* adapting to hypoxia. *M. tb* is an obligate aerobic bacterium, but mounting experimental evidence demonstrates that within granulomas, where O_2 content is below 1% and nitric oxide (NO) content is dramatically elevated, both intracellular and extracellular spaces serve as niches that are colonized by *M. tb* [146, 170–172]. Microbiological and biochemical studies suggest that *M. tb* cannot replicate under hypoxic conditions; however, by shifting its metabolism, it can survive as non- or slowly replicating for decades when oxygen content is at even below 0.06% (NRP phase 2 in Wayne in vitro hypoxia model) [26, 173]. Genetic studies showed upregulation of proline dehydrogenase in NRP *M. tb* adapted to nutrient starvation [154, 174, 175]. Proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase are involved in proline degradation to biosynthesize glutamate and transfer four electrons to the ETC. In these steps, FAD is reduced to $FADH_2$, which is coupled to the reduction of NAD^+ to NADH, an important electron donor for NRP *M. tb*.

M. tb modulates oxidase expression to adapt its ETC according to varying oxygen availability [176, 177]. Under low oxygen conditions and high NO content, while transitioning into NRP state, cytochrome bd oxidase expression is upregulated [174]. Transcriptomics of intracellular *M. tb* showed downregulation of Ndh1, ATP synthase, and menaquinol-cytochrome c oxidase compared to those of replicating counterparts to affirm less demand for generation of energy in the NRP state. On the other hand, nitrate oxidase, Ndh2, and fumarate reductase were shown to be upregulated in the NRP state, suggesting that NRP *M. tb* maintains the ETC and OXPHOS by shifting the functional activity of its components. Sustaining ETC activity is critical for energy generation and recycling of NAD^+ in the NRP state.

Alternate electron acceptors are required for NRP *M. tb* metabolism when O_2 is not present. Nitrate (NO_3) and fumarate have been studied as potential alternatives for NRP *M. tb* electron acceptors, but none of them could support the *M. tb* growth (Fig. 2) [83, 152, 178, 179]. This finding suggests that in the absence of O_2, the ETC in NRP *M. tb* plays a more critical part in redox homeostasis and disposal of reducing equivalents. Biosynthesis of NO_3 by oxidation of NO and NarK mediates the import of NO_3. Intriguingly, the expression of *narK2* and *narU* is upregulated in anaerobic conditions to secure an alternative electron acceptor. Transcriptional

control of *narK2* is regulated by the DosR/DevR dormancy regulon, which is influenced by environmental changes in O_2, NO, and CO levels. The *M. tb* genome contains the *narGHJI* (Rv1161-Rv1164) operon, which encodes a putative membrane-bound nitrate reductase complex. Expression of this operon is not upregulated under hypoxic or nutrient starvation conditions in *M. tb*. An alternate role of NarGHJI is to maintain the redox balance in NRP *M. tb* adapted to hypoxia. Sohaskey et al. have shown that NO_3 helped *M. tb* rapidly adapt to oxygen depletion [43, 180, 181].

Recent metabolomics studies using ^{13}C tracing experiments showed a TCA cycle shift in NRP *M. tb* induced by hypoxia, provoking the biosynthesis of extra succinate as a product of active secretion [142, 152]. The overproduction and secretion of succinate are essential to maintain the NRP viability by acting as an anaplerotic precursor to downstream TCA cycle intermediates and serving as an electrogenic process to maintain membrane potential and ATP biosynthesis (Fig. 2). The essentiality of succinate for membrane bioenergetic function in NRP *M. tb* was proved by in vitro and mouse model studies of succinate dehydrogenase (Sdh1), the membrane anchor for the second ETC component [179, 182–185]. The essentiality of ETC, membrane bioenergetics, and accompanied redox homeostasis is supported by the clinical outcomes of BDQ, delamanid (OPC-67683), and pretomanid (PA-824), which cause ETC poisoning by the accumulation of NO from deazaflavin dependent nitroreductase (Ddn) activity, thereby effectively killing NRP *M. tb* [146, 186–189]. Similar antimicrobial activity targeting NRP *M. tb* with the aforementioned compounds has also been observed in both animals and humans [190] (Table 2).

2.2.3 Exiting from the Non-Replicating Quiescent State

Carbon Sources

Integration of molecular typing with epidemiological studies proved that in most cases, the same strain was responsible for both initial infection and disease relapse found in low-TB burdened areas, suggestive of reactivation. On the other hand, reinfection was implicated in higher-TB burdened countries where various strains were associated with disease relapse [191–193]. Latently infected hosts will produce a positive TB skin test, although they have no clinical symptoms. These latently infected individuals that are immune-competent have a 10% risk of disease reactivation during their lifetime. This chance increases in individuals with a compromised immune system. Common examples of immune systems impairments include co-infection with HIV and/or treatment with immunosuppressive agents, such as TNF-neutralizing drugs. An estimated one-fourth of the world population is latently infected, a huge reservoir for possible reactivation [194, 195].

Some useful in vivo reactivation models of *M. tb* include rabbits and guinea pigs. Cost-effective in vitro experimental strategies have been attempted; nonetheless, knowledge about NRP reactivation and underlying mechanisms for NRP transition to replicating state is limited. Most multiomics studies of reactivation have employed the NRP *M. tb* adapted to hypoxia [196, 197]. Transcriptomic analysis

using this process showed that reactivation altered several pathways, such as restoration of DosR regulon-mediated transcriptional remodeling, DNA repair, ETC activity, and cell wall biosynthesis. Interestingly, a recent biochemical study provoking NRP *M. tb* reactivation by K^+-limiting conditions showed a transcriptional activation in two stages:

i. initial de novo mRNA synthesis, activating cell defense mechanisms and lipid metabolism;
ii. secondary active cell proliferation through inducing central metabolism reactions [198].

Although we intuitively assume the transition from NRP state to active replication is reflected by resuming bacterial growth, we cannot assume the metabolic activities required to exit from the NRP state should be identical to those required in the replicating state. Two recent metabolomics studies showed accumulation of glycolysis, aminosugar biosynthesis, and PPP intermediates in NRP *M. tb* adapted to hypoxia with a decrease in the upstream disaccharide, trehalose [34, 143]. These accumulations were later proven to promote de novo peptidoglycan synthesis for opportunistic reactivation (Fig. 3). This study demonstrated that the accumulation of these metabolites acts as an anticipatory metabolic action to provide a sufficient carbon source required for successful reentry into the normal cell cycle.

A separate metabolomics study of NRP *M. tb* under hypoxia also validated the role of succinate as a potential carbon source used to exit the NRP state (Fig. 3) [142, 152]. Some of the unused succinates are stored, which may later be used to quickly resume carbon flow downstream of the TCA cycle accompanied with NADH and ATP synthesis upon reaeration. Since the succinate/fumarate redox couple midpoint potential is almost neutral ($\varepsilon^{0`} = +0.03$ V), it can help redox balance by fermentation product accumulation through fumarate reductase. Succinate serves as the bridge between fermentative and oxidative metabolic states by its wide range of ability to maintain ATP synthesis and membrane potential and feed the TCA cycle.

Potential Antibiotic Targets Preventing Reactivation of Non-Replicating *M. Tuberculosis*

Succinate Dehydrogenase

Succinate oxidation is coupled to quinone reduction by succinate dehydrogenases (Sdh). Sdh assists in the biosynthesis of TCA cycle intermediates and the generation of the PMF. There are two Sdh complexes found in the *M. tb* genome: Rv0249c-Rv0247c (Sdh1) and Rv3316-Rv3319 (Sdh2) [179, 182]. Sdh complexes have been an attractive target for antifungal agents, including oxadiazole carbohydrazide and thiazole carboxamide [199, 200]. Recent metabolomics and biochemical studies showed that 3-nitropropionate (3NP) is an inhibitor of *M. tb* Sdh by targeting the dicarboxylate-binding site of the subunit A [122, 142]. Treatment of 3NP did not cause a reduction but rather an accumulation of succinate in the TCA cycle, confirming that 3NP is a specific Sdh inhibitor and not an ICL inhibitor.

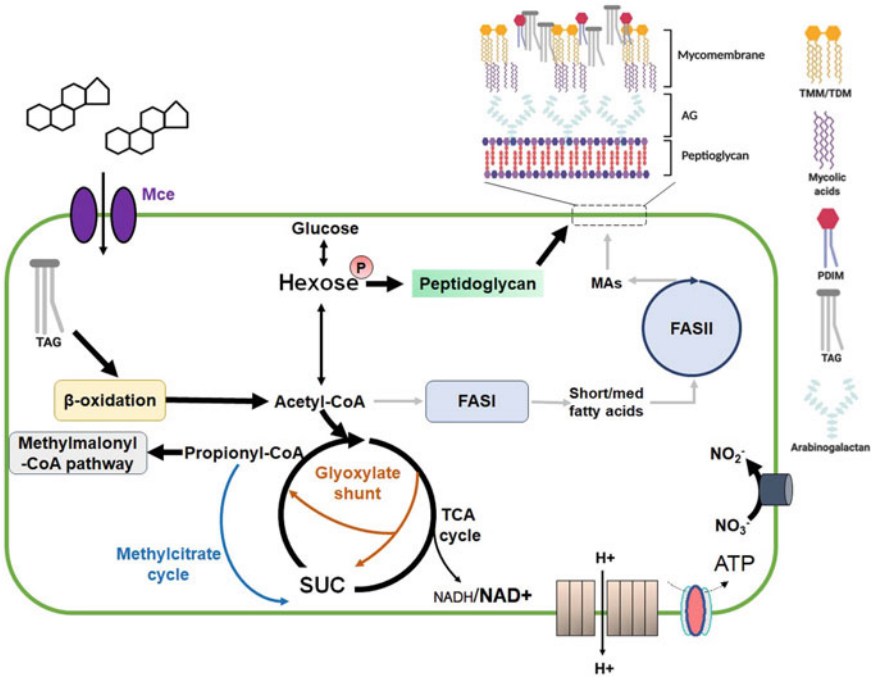

Fig. 3 Initial carbon source candidates required for *M. tb* reactivation. Some of the unused succinates facilitate the resumption of carbon flow through the TCA cycle and accompany biosynthesis of NADH, leading to the generation of proton motive force and ATP. *M. tb* also uses intermediates from the aminosugar biosynthesis pathway as substrates of MurA, the rate-limiting step of peptidoglycan biosynthesis. Other than succinate and aminosugars, triacylglycerol (TAG) is also a potential carbon source used by *M. tb* to exit the NRP state. (*Created with BioRender.com*)

Treatment of 3NP in NRP *M. tb* adapted to hypoxia resulted in a time-dependent delayed regrowth upon reaeration, suggesting the functional essentiality of accumulated succinate as an initial carbon source required for exit from the NRP state. Sdh is an appealing potential drug target to prevent *M. tb* reactivation.

UDP-N-Acetylglucosamine 1-carboxylvinyltransferase (MurA)

A metabolomics study using hypoxic *M. tb* identified accumulation of a discrete set of intermediates in the early portion of glycolysis and aminosugar biosynthesis pathway, including glucose phosphates and UDP-N-Acetyl Glucosamine (UDP-GlcNAc) [143]. During reaeration, following metabolic labeling with ^{13}C-acetate during hypoxia, only the unlabeled fraction of foregoing intermediates exhibited time-dependent depletion with reciprocal induction of UDP-N-Acetyl Muramic acid (UDP-MurNAc) by condensing UDP-GlcNAc with newly synthesized PEP. This is the first committed step of de novo peptidoglycan biosynthesis that is catalyzed by MurA [201]. These findings link the hypoxia-induced

accumulation of glycolysis and aminosugar biosynthesis pathway intermediates to carbon substrates used to reinitiate peptidoglycan biosynthesis of *M. tb* upon reaeration. MurA serves as a potential drug target to block the reactivation of NRP *M. tb* because it catalyzes a critical step in the anticipatory metabolic response.

3 Conclusion

Metabolic remodeling promotes *M. tb* pathogenesis through multiple and complicated operations that go far beyond the energy generation and macromolecule biosynthesis required for replication. These include biochemical regulatory functions to maintain viability such as nutritional homeostasis, membrane bioenergetics, allosteric regulation, antioxidant activity, and extrinsic factors required for survival, such as antibiotic tolerance and host immune interaction. Accumulating evidence supports that metabolic remodeling leads to cellular adaptation in a phenotype-specific process. It is important to recognize that metabolic remodeling is responsible for the diverse phenotypic roles in *M. tb* pathogenesis. The advent of integrated multiomics technologies allowed direct measurements, replacing the indirect inferences made from sequence homology methods. Not only is the knowledge of *M. tb* pathogenesis greatly expanded by these technologies, but more complex questions can be addressed through the interpretation of vast datasets using computational bioinformatics-based modeling. For example, the pharmacological objective is to target metabolically and phenotypically heterogenous *M. tb* populations containing bacilli from replicating to NRP states and subsequent reactivation. Working towards the answers, it is imperative to recognize conceptually novel approaches to identify previously unprecedented new TB interventions.

Core Messages

- Population diversity is an adaptive strategy to environmental changes by metabolic flexibility and mutual interaction.
- Enhancing phenotypic heterogeneity is a bet-hedging tactic against intact immune systems and antibiotic stresses.
- The phenotypic diversity of *M. tb* can be monitored by genomics, transcriptomics, proteomics, and metabolomics.
- Multiomics integration can provide a much more powerful comprehensive knowledge than using single omics alone.
- Multiomics analysis of *M. tb* metabolism aids in novel TB therapeutics discovery to cure drug-resistant TB patients.

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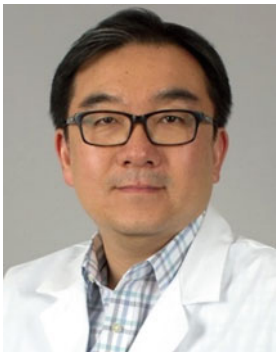
Jae Jin Lee received his Ph.D. in 2015 from Myongji University, Republic of Korea. During his Ph.D., he worked on antimicrobials, multidrug resistance and biochemistry and crystal structure of ESBLs (extended-spectrum beta-lactamases) and carbapenemases. In 2016, he moved to the University of Southern California to work as a postdoctoral research associate in Dr. Hyungjin Eoh's Lab. He has been focused on finding how M. tuberculosis remodels its cell wall by turnover/internalization mechanisms that fuel downstream pathways essential for M. tuberculosis viability under stress conditions (hypoxia, nutrient starvation, and antibiotic treatment) using LC-MS mediated metabolomics. His groundbreaking research on cell wall component-associated metabolic remodeling under stress was published in the top biology journal. Currently, He is researching to create new therapeutic strategies using lipidomics, transcriptomics, and metabolomics to define phenotypic drug-resistance mechanisms.



Hyungjin Eoh earned his DVM degree in Seoul National University, Republic of Korea, and a Ph.D. degree in Colorado State University, USA. Prof. Eoh studied in Weill Cornell Medicine, Cornell University as a postdoctoral research associate. He applied LC-MS mediated metabolomics to elucidate M. tuberculosis metabolic topology while adapting to adverse environmental stresses and redefining functional essentialities of new antibiotic targets. Prof. Eoh established his independent laboratory at the Dept. of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California. In his laboratory, he has expanded his multiomics expertise to study various infectious diseases such as viral infection-induced cancers, Candidiasis, Leprosy, and recently COVID-19, as well as tuberculosis. For these researches, Prof. Eoh has collaborated with various national and international research groups and over 30 research articles in top-tier journals.