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Central carbon metabolism in *Mycobacterium tuberculosis*: an unexpected frontier

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Abstract

Recent advances in liquid chromatography and mass spectrometry have enabled the highly parallel, quantitative measurement of metabolites within a cell and the ability to trace their biochemical fates. In *Mycobacterium tuberculosis* (Mtb), these advances have highlighted major gaps in our understanding of central carbon metabolism (CCM) that have prompted fresh interpretations of the composition and structure of its metabolic pathways and the phenotypes of Mtb strains in which CCM genes have been deleted. High-throughput screens have demonstrated that small chemical compounds can selectively inhibit some enzymes of Mtb's CCM while sparing homologs in the host. Mtb's CCM has thus emerged as a frontier for both fundamental and translational research.

Second looks and second thoughts

Few would challenge the importance of central carbon metabolism (CCM) - defined here as the enzymatic transformation of carbon through glycolysis, gluconeogenesis, the pentose phosphate shunt and tricarboxylic acid (TCA) cycle - to the physiology of a bacterium. After all, seminal studies at the Carnegie Institute over 50 years ago demonstrated that the same metabolic reactions and pathways found in mammals are present in *Escherichia coli* [1]. When it later emerged that the genes and enzymes encoding these reactions were also conserved [2], many came to view CCM as an invariant set of reactions carried out by enzymes whose identities in a given organism can be revealed by bioinformatic analysis of its genome. One could thus question whether further research on CCM is either interesting or necessary.

For *M. tuberculosis* (Mtb) however, matters could not be more different. Mtb is the etiologic agent of the tuberculosis (TB) pandemic and leading bacterial cause of deaths worldwide. Unlike most pathogens, Mtb has humans as its only known reservoir and the macrophage phagosome is its chief locale [3–6]. Mtb has thus evolved within an ultra-narrow ecologic niche, apart from other microbes. In addition, growing evidence has implicated Mtb's CCM

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as a key determinant of its pathogenicity [7, 8]. Mtb's CCM thus appears to have evolved to serve interdependent physiologic and pathogenic roles.

Recent work, much of it using new tools, has revealed distinctive elements of Mtb's CCM that have helped to affirm this view. Of greater significance however, these same studies have exposed even greater gaps in our knowledge of the basic biochemical composition and organization of CCM in Mtb. Mtb's CCM has thus re-emerged as an uncharted frontier in microbial physiology and pathogenesis. Below we review some of the key findings that have informed this view, present an experimentally annotated view of Mtb's CCM, and discuss potential basic and translational implications of this turnabout.

Genomics: clarity and confusion

Interest in Mtb's CCM grew from Dubos' efforts to develop a medium to support its maximal rate of growth *in vitro*. Recognition that CCM in Mtb might be distinctive stemmed from the finding by Segal and Bloch that Mtb recovered from the lungs of infected mice preferentially metabolized fatty acids over carbohydrates *ex vivo* [9]. Biochemical studies of Mtb extracts [10], however, provided only fragmented views of its metabolic network.

A more global picture of Mtb's CCM emerged with the publication of its genome [11]. Homology-based sequence analyses and comparative metabolic modeling enabled *in silico* inventorying of all genomic orthologs of canonical CCM enzymes in Mtb [11–14]. This was soon followed by bioinformatic comparisons that revealed important differences in the CCM genome of Mtb from that of the closely related pathogen *Mycobacterium bovis* (from which the vaccine strain BCG was derived), whose *glpK*-encoded glycerol kinase and *pykA*-encoded pyruvate kinase have been mutationally inactivated and which requires pyruvate for growth on glycolytic carbon sources [27]. Transcriptional profiling studies subsequently revealed extensive remodeling of the expression of Mtb's CCM genes during host infection that presumably reflect its metabolic adaptation to the host niche [6, 20]. Mutation studies identified genes only conditionally required for *in vitro* growth but whose disruption led to some of the most profound attenuations of Mtb yet reported in mice or guinea pigs [7, 8]. Among these mutants were Mtb strains lacking isocitrate lyase (Icl, encoded by *icl1* plus *icl2*), phosphoenolpyruvate (PEP) carboxykinase (PEPCK, encoded by *pckA*) and lipoamide dehydrogenase (Lpd, encoded by *lpdC*) [6, 15–19], and a strain lacking dihydrolipoamide acyltransferase (DlaT) that was severely attenuated in the guinea pig [6, 20–22]. Such studies thus provided further affirmation of the distinctive structure and composition of Mtb's CCM.

At the same time, other experiments began to blur this picture. Eight genes annotated as encoding the subunits of Mtb's pyruvate dehydrogenase complex (PDH; *pdhA*, *pdhB*, *pdhC*, and *aceE*), which serves as the metabolic junction between glycolysis and the TCA cycle, and α -ketoglutarate dehydrogenase (KDH; *sucA* and *sucB*), a canonical TCA cycle enzyme complex, including two orthologs of the common E3 subunit [lipoamide dehydrogenase (Lpd; *lpdA* and *lpdB*)]. However, only one gene, *aceE*, fulfilled its predicted role, which was to serve as the E1 component of Mtb PDH [11, 23–27] (Figure 1; Supplementary Table S1). *sucB* proved to be part of PDH rather than KDH [22], and biochemical and metabolomic analyses demonstrated a lack of KDH activity in Mtb under the conditions studied [19, 22, 25, 26, 28]. Mtb's functional Lpd (encoded by Rv0462, re-annotated as *lpdC*) was found to function not only as the E3 subunit of Mtb's PDH and branched chain ketoacid dehydrogenase (BCKADH) complexes [19, 23] but also as E1 of an NADH-dependent peroxynitrite reductase/oxidase [24]. The other genes originally annotated as constituting the E1 (*pdhA* and *pdhB*) and E2 (*pdhC*) of PDH instead encode the E1 and E2 of BCKADH [19]. Metabolomic studies revealed that the gene annotated as the E1 of KDH (*sucA*;

Rv1248c) catalyzes a reaction between two intermediates of CCM (α -ketoglutarate and glyoxylate), producing a previously undescribed metabolite of Mtb, hydroxyoxoadipate, whose function remains to be discovered [19, 28].

Moreover, homology-based predictions have not sufficed to resolve the enzymatic, metabolic and regulatory specificities of genes with multiple paralogs. Mtb encodes paralogs for many pathways in CCM. For example, while both *glpX* and *Rv2131c* are annotated as fructose biphosphatases, each exhibits a distinct, though overlapping, range of substrate specificities. By contrast, Mtb also encodes two phosphofructokinases (*pfkA* and *pfkB*), each of which exhibits a distinct expression pattern, while the function and activity of its two annotated isocitrate lyases (*icl1* and *icl2*, both of which serve as isocitrate and methylcitrate lyases) appear to be redundant [11, 18, 27, 29–33].

Homology-based approaches have similarly failed to suggest a function for nearly 40% of microbial genes, including those of Mtb [11]. Reciprocally, up to 30% of detected enzymatic activities have not been ascribed to a known gene [34]. For example, the type I metal ion-independent fructose biphosphate aldolase (Fba) representing the predominant Fba activity in Mtb fermentor cultures is an orphan enzyme of Mtb's CCM whose gene remains to be identified [35, 36]. The sizeable proportion of unidentified and/or unannotated metabolic enzymes in Mtb thus poses a potentially significant challenge for modeling-based studies.

Finally, genome-based approaches have left the connectivities of metabolic pathways, the directionalities of reactions, the fluxes of metabolites and many of the regulatory events characteristic of CCM, including negative and positive feedback by substrates and products, wholly unaddressed. A related question also left unanswered by genomics is what functions Mtb has evolved its CCM to optimize. *E. coli*, for example, has optimized metabolic fluxes to support maximal growth per unit carbon consumed, perhaps reflecting the diverse and polymicrobial niches within which it resides. Mtb, by contrast, occupies an ultra-narrow host niche, within which it is believed to reside for prolonged intervals in a non- or slowly-replicating state with reduced ATP pools, but it also undergoes brief periods of active replication to transmit itself from one host to another [37]. Experimental knowledge of the metabolic requirements accompanying Mtb's natural life cycle is lacking. Two cardinal contributions of genome-enabled analyses of CCM have thus been to highlight major deficiencies in accompanying biochemical knowledge and to challenge canonical models of CCM in Mtb. A genomically annotated inventory of known or homology-predicted CCM enzymes in Mtb is presented in Figure 1 and Supplementary Table S1.

Enter metabolomics

Metabolomics—ideally, the simultaneous measurement of all metabolites in a biological system under a given set of conditions and the ability to trace their transformations—provides a powerful tool with which to characterize a cell's steady state and its adaptations to perturbation [38–40]. Metabolites represent the integrated product of a cell's genome, proteome and environment and the most direct reporters of a cell's metabolic state. Recent advances in sample preparation, liquid chromatography and mass spectrometry have made it feasible to study many metabolites quantitatively and in parallel on a time scale compatible with the rapid turnover of many intermediate metabolites. Moreover, the use of isotopically labeled metabolites has extended these methods to allow metabolic tracing [41]. Though nascent, the application of such tools to Mtb's CCM is already delivering surprising insights.

It was mentioned above that a metabolomic analysis led to reassignment of Rv1248c – originally annotated as the E1 component of α -ketoglutarate dehydrogenase complex – as a hydroxyoxoadipate synthase (HOAS) [28]. Previous studies revealed that Mtb lacked α -ketoglutarate dehydrogenase activity, due to the absence of a functional E2,

dihydrolipoamide succinyltransferase, and that recombinant Rv1248c could nonoxidatively decarboxylate α -ketoglutarate to succinic semialdehyde (SSA) which, in turn, could be converted to succinate by the SSA dehydrogenases GabD1 and GabD2. Subsequent ^1H -NMR experiments, however, found that SSA formation did not occur at a rate commensurate with the proposed metabolic role and was a likely slow side reaction of Rv1248c. Its true physiologic activity was instead discovered with the development of a new methodologic approach, termed activity based metabolite profiling, in which a purified recombinant enzyme of uncertain function is incubated with a small-molecule cell extract and reaction progress monitored for the consumption and production of potential substrates and products by high resolution liquid chromatography-mass spectrometry. Thus, perhaps just as important as the discovery of the specific activity of Rv1248c, was the methodologic approach and its potential to biochemically identify additional yet unannotated components of Mtb's CCM.

Another early impact of metabolite profiling and carbon tracing studies was to define a metabolically indispensable role for PEPCK in Mtb. PEPCK was predicted to catalyze the reversible interconversion of oxaloacetate (OAA) and PEP. Genetic studies established its essentiality for both *in vitro* growth on fatty acid carbon sources and survival in mice [16, 17]. However, comparison of uniformly ^{13}C labeled acetate carbon flux in wild-type and *pckA*-deleted Mtb established that PEPCK predominantly catalyzes the unidirectional conversion of OAA into PEP. While pyruvate was still produced from TCA cycle intermediates in the absence of PEPCK, presumably via the malic enzyme, it was not further metabolized into PEP [16, 17], indicating a lack of a pyruvate phosphate dikinase (*ppdK*) activity. Thus, PEPCK is essential for gluconeogenesis in Mtb and gluconeogenesis is essential for the pathogenesis of Mtb in mice.

Yet another early application of metabolomics combined metabolite pool size measurements and isotopic tracing to reveal that carbon flow between the TCA cycle metabolites α -ketoglutarate and succinate was discontinuous during *in vitro* logarithmic growth [41]. This confirmed that Mtb lacks a canonical KDH under those conditions and suggested that Mtb operates a variant or bifurcated TCA cycle [10, 42]. Strikingly, recent metabolomic studies of another human pathogen, *Plasmodium falciparum*, revealed that it has similarly bifurcated its TCA cycle at α -ketoglutarate into two branched pathways that enable it to metabolize glutamate and/or glutamine into two-carbon units in a redox-neutral manner [43]. The directionality of a recently identified anaerobic-type aerotolerant α -ketoglutarate:ferredoxin oxidoreductase (KGFOR) and the conditions under which it could restore an intact TCA cycle in Mtb thus await resolution [44].

The initial metabolomic study of Mtb's CCM [41] further revealed that, unlike other bacteria, Mtb can simultaneously co-catabolize multiple carbon sources to enhance its growth by directing individual component carbon sources to distinct metabolic fates ('compartmentalization'). These studies further showed that this functional 'compartmentalization' was also associated with the 'segregated' metabolism of each carbon source from the other, such that individual carbon sources could be simultaneously metabolized in opposite directions through the same pathway [41].

In sum, these examples established that the CCM of Mtb involves anomalous arrangements of known metabolic pathways, including a discontinuous TCA cycle and impaired gluconeogenic conversion of pyruvate into PEP; novel metabolic reactions of CCM intermediates, such as the production of HOA from α -ketoglutarate and glyoxylate; and unprecedented regulatory features, including co-catabolism of multiple carbon sources in both a functionally compartmentalized and biochemically segregated manner. An experimentally revised view of Mtb's CCM pathways is presented in Figure 2.

Challenges in translation

While *in vitro* studies have brought Mtb's CCM into a new focus, these same studies have simultaneously begun to challenge existing views of Mtb's niches in the host and its metabolic states within them. Efforts to study Mtb metabolism while infecting a host have depended largely on transcriptional profiling studies [6, 20, 45–48] and the impact of gene knockouts on Mtb virulence or survival in animals. For example, as noted, Mtb strains lacking a functional isocitrate/methylisocitrate lyase or PEPCK were profoundly attenuated during the acute and chronic phases of infection in a mouse model of pulmonary tuberculosis [15–18]. That Mtb depends on lipids during host infection was further suggested by the inability of Mtb mutants lacking either the *mce4*-encoded cholesterol transporter or cholesterol-degrading enzyme HsaC to persist during the chronic phase of infection in the lungs of mice and guinea pigs, respectively [49, 50]. The phenotypes of auxotrophic mutants in mice further suggested that Mtb can salvage host niacin for NAD cofactor biosynthesis but must synthesize its own leucine, methionine, tryptophan, arginine, pantothenic acid (vitamin B5), vitamin B6 and biotin [7, 51–56].

However, emergent metabolic knowledge has made these studies unexpectedly complex to interpret in mechanistic terms. For example, both the isocitrate lyase and methylcitrate lyase activities are catalyzed by a single active site. It is thus unclear whether compromise of the glyoxylate shunt and/or methylcitrate cycle accounts for the profound attenuation of Icl-deficient Mtb. While loss of isocitrate lyase activity is expected to result in the inability to incorporate carbon from fatty acids (a form of starvation), simultaneous loss of methylisocitrate lyase activity is predicted to result in the potential accumulation of toxic propionyl Coenzyme A (CoA) metabolites (a form of intoxication) [57–59]. Interpretation of the phenotype of mutants unable to synthesize pantothenic acid or vitamin B6 is similarly confounded by the potential roles of these co-factors in anti-oxidant defense as well as CCM [52, 55]. Death of the PEPCK mutant in infected mice is unexplained and could reflect secondary metabolic perturbations that sensitize Mtb to stresses encountered within the host. The recently reported attenuation of an Mtb strain lacking the LpqY-SugA-SugB-SugC-encoded trehalose recycling transporter in mice similarly leaves the role of Mtb carbohydrate metabolism unresolved. This transporter was shown to be the sole transporter capable of reclaiming trehalose, a sugar not found in mammals, but instead released during turnover of the cell wall glycolipid, trehalose monomycolate. Surprisingly, LpqY-SugA-SugB-SugC-deficient Mtb exhibited no defects in cell wall composition [60]. The biochemical, and potentially metabolic, fate of trehalose thus remains unresolved. Disruption of *lpdC* resulted in one of the most profound attenuations of Mtb yet reported in mice, far greater than disruption of dihydrolipoamide acyltransferase (DlaT) [22], which similar to Lpd is a component of PDH and peroxynitrite reductase/peroxidase (PNR/P) [21, 24]. As noted, Lpd also constitutes a component of Mtb's BCKADH, a function not shared with DlaT. Does Mtb require branched chain amino acids as substrates, or is Mtb poisoned by the marked, sustained intracellular accumulation of pyruvate, branched chain amino acids and branched chain α -keto acids observed *in vitro* in the absence of Lpd [19]? Indeed, deletion of the *glgE*-encoded maltosyl transferase led to attenuation of virulence associated with intoxication by the accumulating substrate, maltose-1-phosphate [61]. Thus, while Mtb mutants currently represent the most powerful bioprobes with which to investigate its CCM during infection, interpretation of their phenotypes remains incomplete and awaits accompanying biochemical analyses.

Targeting CCM for antimycobacterials

The profound attenuation associated with mutations of some of the enzymes in Mtb's CCM commends these enzymes as potential drug targets. Among the potential advantages are the

opportunity to design inhibitors based on transition state analogues, as was achieved against *P. falciparum*'s purine nucleoside phosphorylase [62, 63], and evidence that CCM enzyme active sites are often sub-saturated making them potentially more susceptible to inhibitors [64, 65]. Enthusiasm for developing CCM enzymes as potential drug targets however has been heavily muted by the presence of orthologous host enzymes. Yet, evidence is growing that Mtb enzymes can be inhibited while sparing their human counterparts. These include two broad spectrum antibiotics in widespread clinical use, fluoroquinolones, which selectively inhibit bacterial DNA gyrases and are active against Mtb, and trimethoprim (TMP), a selective inhibitor of bacterial dihydrofolate reductases (DHFR) that includes Mtb's DHFR but lacks whole cell activity. While the structural basis for the selectivity of fluoroquinolones remains unresolved, the selectivity of TMP was shown to be due to a highly cooperative binding property specific to bacterial DHFRs for TMP and the NADPH cofactor [66]. Among TB drugs, an especially remarkable example of bacterial selectivity that is currently in clinical trials is the diarylquinoline, TMC207. TMC207 potently kills both replicating and non-replicating Mtb and inhibits the c subunit of its ATP synthase [67–71]. Remarkably, this inhibition is associated with a selectivity factor of 20,000 over that for the closely related human mitochondrial ortholog, through what could be as little as a single amino acid difference in the membrane-spanning binding pocket for TMC207 [69]. Other examples of antimycobacterials at earlier stages of development include rhodanines that inhibit DlaT, triazospiridimethoxybenzoyls that inhibit Lpd and oxathiazol-2-ones that inhibit the proteasome—in each case targeting the Mtb enzyme while sparing its human ortholog [21, 72, 73]. Together, these examples allay the concern that targets in Mtb cannot be inhibited with sufficient selectivity to avoid mechanism-based toxicity to the host.

A broader conceptual challenge associated with advancing enzymes of Mtb's CCM as potential drug targets is defining the criteria required for their validation. Most drugs, including antibiotics, work through the stoichiometric inhibition of an intracellular biochemical target. However, growing evidence indicates that metabolic enzymes can exist at levels much higher than those needed to support growth and/or viability. Not all genetically essential enzymes are therefore likely to represent equally viable drug targets [74]. In addition, most enzymes of CCM serve multiple interconnected pathways that are themselves subject to robust homeostatic regulation [65]. Validation of Mtb's CCM enzymes as potential drug targets is thus likely to require biochemical knowledge of the specific metabolic pathways that define the genetic essentiality of a given enzyme as well as the quantitative level of inhibition needed to achieve growth inhibition or death of Mtb.

Concluding remarks

In evolution, conservation emerges from, without extinguishing, diversity. The CCM of microbes appears to serve the specific niches in which they reside. For Mtb, the study of CCM thus represents a potential window into its pathogenicity and the selective pressures encountered in its ecologic and nutritional niches, as well as a guide to new therapeutics. However, direct biochemical knowledge of Mtb's CCM remains quite limited. Here, we stressed how little we understand with respect to paralogous enzymes, the physical and functional organization of Mtb's metabolic enzymes and pathways, the source and fate of α -ketoglutarate and directionality of flow in the TCA cycle in Mtb. Thus, the more Mtb's CCM is studied, the more distinctive adaptations are likely to be revealed.

An even bigger gap in knowledge is that what we do know is based largely on Mtb growing in oxygen-rich, nutrient-replete conditions that do not mimic the conditions within the host. Metabolome-wide knowledge is almost completely lacking about Mtb at different levels of hypoxia; non-replicating Mtb; Mtb competing with the host for iron; Mtb subsisting on physiologic diets that remain to be defined in its diverse niches; Mtb under oxidative,

nitrosative or other immune attack; or Mtb during drug treatment. Furthermore, not all CCM metabolites are accessible by current methods.

Another key area awaiting experimental answers pertains to questions of regulation. What controls Mtb's CCM besides transcription and substrate/product feedback? Are kinases and other regulatory proteins critical, as in eukaryotes? And how do these mechanisms relate to Mtb's nutrient transport systems? Do microRNAs or other processes control the level and lifespan of CCM enzymes? A more general question of potentially therapeutic significance is how a robust metabolic network with multiple interconnected nodes and facile bidirectional reactions responds to perturbation.

Beyond our interest in Mtb's basic physiology, if we are to develop therapeutics that are more rapidly effective against Mtb in the human host we urgently need the ability to assess the metabolic state of Mtb as it exists in its diverse pathologic sites. To apply metabolomics to this challenge will require solving key problems in sample acquisition, storage and shipment and improving assay sensitivity. Only when such information is coupled with deeper biochemical understanding, can we hope to correctly predict the extent and scope of metabolic alterations associated with deletion of a given gene of CCM and the impact of various levels of inhibition of the enzyme it encodes. CCM has thus re-emerged as an unexpected frontier in microbial physiology and clinical medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Bioinformatic inventory of the CCM network in Mtb. Pathway schematic depicting a bioinformatic reconstruction and inventory of Mtb's CCM pathways. Abbreviations: CO₂, carbon dioxide; CoA, coenzyme A; GABA, gamma-aminobutyrate.

Figure 2.

Experimentally annotated view of the CCM network of Mtb. Solid blue arrows denote biochemically confirmed reactions using purified recombinant enzymes; solid black lines denote biochemically confirmed reactions using Mtb protein extracts; dotted gray arrows denote genetically or bioinformatically inferred reactions; yellow highlighting indicates metabolomically detected reactions. Asterisk denotes multiple reactions not shown. Dagger denotes inferred detection of oxaloacetate using aspartate as a surrogate reporter that is thought to exist in rapid equilibrium with oxaloacetate. Abbreviations: CO₂, carbon dioxide; CoA, coenzyme A.