The anaplerotic node is essential for the intracellular survival of Mycobacterium tuberculosis

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Enzymes at the phosphoenolpyruvate (PEP)—pyruvate—
oxaloacetate or anaplerotic (ANA) node control the metabolic
flux to glycolysis, gluconeogenesis, and anaplerosis. Here we
used genetic, biochemical, and 13C isotopomer analysis to char-
acterize the role of the enzymes at the ANA node in intracellular
survival of the world’s most successful bacterial pathogen, Mycobacterium tuberculosis (Mtb). We show that each of the
four ANA enzymes, pyruvate carboxylase (PCA), PEP carboxy-
kinase (PCK), malic enzyme (MEZ), and pyruvate phosphate dikin-
ase (PPDK), performs a unique and essential metabolic function
during the intracellular survival of Mtb. We show that in addition
to PCK, intracellular Mtb requires PPDK as an alternative gateway
to gluconeogenesis. Propionate and cholesterol detoxification
was also identified as an essential function of PPDK revealing an
unexpected role for the ANA node in the metabolism of these phys-
ically important intracellular substrates and highlighting this
enzyme as a tuberculosis (TB)-specific drug target. We show that
anaplerotic fixation of CO2 through the ANA node is essential for
intracellular survival of Mtb and that Mtb possesses three enzymes
(PCA, PCK, and MEZ) capable of fulfilling this function. In addi-
tion to providing a back-up role in anaplerosis we show that MEZ
also has a role in lipid biosynthesis. MEZ knockout strains have an
altered cell wall and were deficient in the initial entry into macro-
phages. This work reveals that the ANA node is a focal point for
controlling the intracellular replication of Mtb, which goes beyond
canonical gluconeogenesis and represents a promising target for
designing novel anti-TB drugs.

Mycobacterium tuberculosis (Mtb)2 is the causative agent
of a global tuberculosis epidemic that has now reached stag-
gering levels and is the biggest infectious disease killer
worldwide (1). The limited number of drugs available that
have activity against Mtb, the lengthy multidrug regimen
needed to eradicate the infection, and the worldwide spread
of multi- and extensively drug-resistant strains (2, 3) all com-
plicate the treatment of tuberculosis (TB) and thwart
attempts to control this global emergency. Mtb is an unusual
bacterial pathogen, with the remarkable ability to cause both
acute life threatening disease and also clinically latent infec-
tions that can persist for the lifetime of the human host.
Metabolic reprogramming in response to the host niche during
both acute and chronic phases of TB infections is a cru-
cial determinant of virulence (4, 5). Experimental evidence
has identified central carbon metabolism as instrumental in
this pathogenic strategy (4, 6). Therefore an in-depth knowl-
edge and understanding of the central metabolism both in
vitro and in vivo will expedite the identification and valida-
tion of novel strategies to combat TB.

We previously applied the systems-based tool 13C-flux spec-
tral analysis (13C-FSA) to show that intracellular Mtb co-me-
tabolizes multiple gluconeogenic and glycolytic carbon sub-
strates by utilizing the reactions of the phosphoenolpyruvate
(PEP)—pyruvate—oxaloacetate (OAA) or anaplerotic (ANA)
node, in both the gluconeogenic and carbon-fixing anaplerotic
direction simultaneously (7). The ANA node consists of several
bidirectional reactions, modeling is not able to predict which
genes and reactions are required nor their directionality (8).
The ANA node lies at the heart of the central metabolism inter-
connecting the main pathways of the central metabolism (gly-
colysis, gluconeogenesis, and the TCA cycle) controlling the
distribution of flux between anabolism, catabolism, and energy
supply to the cell (9). At this node the end products of glycolysis,
PEP and pyruvate, either: 1) serve directly as precursors for
anabolism or 2) enter the TCA cycle via acetyl Co-A or
anaplerotic reactions and this node is also the starting point
for gluconeogenesis.

The ANA node of Mtb consists of the enzymes pyruvate
 carboxylase (PCA), PEP carboxykinase (PCK), malic enzyme
(MEZ), and pyruvate phosphate dikinase (PPDK) (Fig. 1). Of
these enzymes, only PCK has been shown to have an essential
role in gluconeogenesis when Mtb is growing on lipid sub-
strates in vitro and for survival in macrophage and murine
models of TB (4) or hypoxia in a chemostat (10). The role of

1 The abbreviations used are: Mtb, Mycobacterium tuberculosis; TB, tuberculo-
sis; PEP, phosphoenolpyruvate; 13C-FSA, 13C-flux spectral analysis; OAA, oxaloacetate; ANA, anaplerotic; PCA, pyruvate carboxylase; PCK, PEP car-
boxykinase; MEZ, malic enzyme; PPDK, pyruvate phosphate dikinase; FA, fatty acid; FAME, fatty acid methyl ester; MAME, mycolic acid methyl esters;
FAS, fatty acid synthase; PDH, pyruvate dehydrogenase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; FCS, fetal calf serum.

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5 The article contains Tables S1–S4 and Figs. S1–S3.

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other members of the ANA node in TB pathogenesis remains unexplored.

In this study we use a combination of genetics and biochemistry to test the redundancy within the ANA node to establish whether targeting its deregulation could inhibit intracellular nutrient acquisition and therefore intracellular growth. We show that in addition to being a gateway into both gluconeogenesis and anaplerosis, the ANA node has unexpected roles in cholesterol and propionate detoxification and lipid synthesis. Despite apparent functional overlap, we show that each enzyme in the ANA node fulfills a distinct metabolic function during the intracellular growth of Mtb and is thereby a potentially fruitful target for drug development.

Results

In silico Genome Scale Metabolic Networks (11–13) indicate that the ANA node of Mtb consists of a set of functionally redundant enzymes (Fig. 1). Nevertheless, the essential role of PCK during gluconeogenesis and intracellular survival (4) demonstrates that at least this enzyme plays a role that cannot be complemented by other ANA enzymes. To define the role of each enzyme in the ANA node we generated single mutant H37Rv strains Δpca, Δpck, Δppdk, Δmez and also a double mutant strain Δpca–Δpck. The growth rates of these mutants were similar to that of the WT strain when cultured in standard Middlebrook 7H9 with glycerol and Tween 80 demonstrating that these enzymes are not required for bacterial growth in rich media (Fig. S1).

The anaplerotic node of Mtb

Figure 1. The PEP–pyruvate–oxaloacetate node or ANA node of metabolism in Mtb. The larger arrows point into the predicted physiological direction.

The anaplerotic node of Mtb

The ANA node is required for intracellular invasion and replication

To test whether the ANA node is important for Mtb during intracellular growth we infected THP-1 macrophages with WT, Δpca, Δpck, Δppdk, Δmez, and Δpca–Δpck (Fig. 2A) and their respective complemented strains (Fig. 2B) of Mtb and monitored intracellular invasion and survival. Deletion of pca had no observable effect on intracellular bacterial growth (Fig. 2A). However, surprisingly deletion of both pca and pck resulted in severe impairment of intracellular survival and loss of the strain from the macrophage (Fig. 2, A and B). As described previously for the Erdman strain of Mtb (4) Δpck H37Rv also had impaired survival but this phenotype was not as severe in comparison to Δpca–Δpck (Fig. 2, A and B). These results demonstrated that PCA is essential for intracellular growth in the absence of PCK.

Δppdk had a similar intracellular growth defect to Mtb strains lacking PCK (Fig. 2A) indicating that this enzyme is also performing an essential role during infection. Although intracellular Δmez replicated as the parental strain this mutant was less efficient at entering macrophages indicating an invasion phenotype (Fig. 2, C and D).

Anaplerosis through the ANA node is essential for intracellular growth

To understand the intracellular phenotypes we next investigated the ability of our ANA enzyme mutants to grow on physiologically relevant carbon sources. PCA was required for growth on pyruvate and substrates that enter metabolism as pyruvate (alanine, serine, and lactate) (Table S1). This phenotype could be complemented by reintroducing the gene into the genome under control of the constitutive hsp60 promoter into the attB site; but could also be complemented by addition of aspartate (as proxy for oxaloacetate, which is labile (14) to the media or by growing the cultures in the presence of 5% CO2 (Table S1). These results demonstrated that PCA is performing an anaplerotic function replenishing oxaloacetate (hence aspartate rescues the mutant) into the TCA cycle when growing on these substrates; but that this role can be complemented when sufficient CO2 is present presumably by the actions of either MEZ or PCK. It has already been shown by us and others that Mtb PCK can operate in the carboxylating (anaplerotic) direction (15, 16), however, the role of MEZ is unknown.

PCK was required for the growth of Mtb H37Rv on all gluconeogenic substrates tested (Tables S1 and S2) including those identified as potential intracellular carbon sources by us and others (acetate, glutamate, asparagine, cholesterol, alanine, and combinations of these substrates and their byproducts) (17). This is in accordance with data for Mtb Erdman, which showed that the gluconeogenic flux of acetate was blocked in the absence of PCK (4). The double Δpca–Δpck mutant grew identically to the Δpck mutant on the media tested, except that this strain was additionally unable to grow on glycerol unless supplemented with 5% CO2 (Table S1) suggesting that, in vitro, MEZ is able to comple-
ment the anaplerotic function of PCA if provided with sufficient amounts of CO$_2$.

To directly test the hypothesis that PCA is performing an anaplerotic function during intracellular growth and that MEZ functions anaplerotically in the absence of both PCA and PCK we infected unlabeled THP-1 macrophages with WT H37Rv, \( \Delta \text{pca} \) and \( \Delta \text{pca–pck} \) in RPMI medium containing sodium \(^{13}\text{C}\) bicarbonate/RPMI and compared the labeling profile of amino acids after 48 h. We previously performed this experiment with \( \Delta \text{pck} \) and showed that this enzyme was contributing to the intracellular fixation of CO$_2$ (7). In the absence of PCA, \(^{13}\text{C}\) incorporation into proteogenic amino acids was significantly reduced as compared with the WT strain demonstrating that this enzyme was also contributing to the fixation of carbon from CO$_2$ during intracellular growth (Fig. 3, Table S3). The results are consistent with the \textit{in vitro} evidence that PCA functions primarily as an anaplerotic enzyme. In the double knockout of PCA and PCK \(^{13}\text{C}\) labeling of amino acids was further reduced as compared with \( \Delta \text{pca} \) but there were still small amounts of \(^{13}\text{C}\) labeling in aspartate and methionine demonstrating that there is an additional enzyme fixing carbon into OAA, presumably MEZ (Fig. 3, Table S3). However, this diminished CO$_2$ fixation correlated with reduced intracellular survival highlighting the importance of anaplerosis through the ANA node for infection.

**MEZ has a role in lipid biosynthesis**

Although \( \Delta \text{mez} \) was dispensable for the growth of \( \text{Mtb} \) H37Rv on all gluconeogenic substrates and glycolytic substrates tested (Tables S1 and S2), the mutant displayed a glossy and viscous morphology on solid media, which combined with the impaired invasion phenotype suggested that this strain may have an altered cell wall lipid profile.

To test whether the altered \textit{in vitro} growth morphology phenotype was due to alterations in the cell envelope lipid compo-
The anaplerotic node of Mtb

sition, we biochemically analyzed the lipid content of WT, \( \Delta \text{mez} \), and the complemented strain \( \Delta \text{mez}:\text{mez} \). We extracted apolar and polar lipids from the strains and analyzed the extracts by 2D thin-layer chromatography (TLC). The strains were initially treated with petroleum ether to extract outer, non-covalently bound lipids, prior to extraction of inner apolar lipids and polar lipids. The \( \Delta \text{mez} \) mutant showed an accumulation of a major species in solvent System C (18) that co-migrated in the same region where free fatty acids (FA) and free mycolic acids (MA) are detected (Fig. 4A, Fig. S2). Subsequently we extracted mycolic acid methyl esters (MAMEs) from apolar lipids (containing trehalose mono- and dimycolates, TMM and TDM, and free FA) and confirmed that \( \Delta \text{mez} \) was accumulating free MA with an accompanying decrease in the levels of cell wall-bound mycolates from delipidated cells (Fig. 4B). This data were further supported by GC-MS analysis, which detected higher levels of C26 species in the \( \Delta \text{mez} \) strain (Fig. S3). Lipid profiles were restored to those of the parental strain on complementation (Fig. 4, Figs. S2 and S3).

Malic enzymes catalyze the reversible oxidative decarboxylation of malate to produce CO\(_2\) and pyruvate with the concomitant reduction of NAD(P\(^+\)) to NAD(P)H. These enzymes vary in their reversiblity and cofactor preference and their role in metabolism is often not clearly established. To validate \( Mtb \) MEZ functional properties we determined the catalytic properties of the purified enzyme (Table 1).

The enzyme was able to decarboxylate malate (forward, gluconeogenic, reaction) using either NAD\(^+\) or NADP\(^+\) with a preference for NAD\(^+\) demonstrating that this enzyme can provide both NADH and NADPH for lipid biosynthesis. The enzyme showed a sigmoidicity at low malate concentrations indicating homotropic allosteric regulation. The reverse reductive pyruvate carboxylation reaction showed typical Michaelis-Menten kinetics confirming that \( Mtb \) MEZ can serve as a backup anaplerotic enzyme for \( Mtb \). However, the substrate affinities and maximum reaction rates show that the forward reaction is preferred.

**PPDK is essential for cholesterol and propionate detoxification**

The combined actions of MEZ and PPDK should be able to provide \( Mtb \) with an alternative gluconeogenic route that circumvents the requirement for PCK (Fig. 1). Strikingly, \( \Delta \text{ppdk} \) was able to grow on all gluconeogenic and glycolytic substrates tested with the exception of medium containing cholesterol as a sole carbon source or a combination of cholesterol and acetate, even though the mutant could grow in acetate as a sole carbon source (Tables S1 and S2). Moreover, addition of cholesterol to minimal media containing pyruvate, glycerol, or glutamate similarly inhibited the growth of \( \Delta \text{ppdk} \) (Table S2). This phenotype has been observed in other mutant \( Mtb \) strains that are unable to metabolize cholesterol and has been linked to the accumulation of toxic propionyl-CoA produced from cholesterol catabolism (19, 20).

Propionate did indeed inhibit the growth of \( \Delta \text{ppdk} \) in standard 7H9 medium (containing glycerol and Tween 80) as compared with the WT or the complemented strain \( \Delta \text{ppdk} \).
**The anaplerotic node of Mtb**

**Table 1**

<table>
<thead>
<tr>
<th>Substrate (concentration range; pH)</th>
<th>Kinetic constants for Mtb MEZ</th>
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<tbody>
<tr>
<td>Gluconeogenic reaction (l-malate decarboxylation)</td>
<td>$K_{m}/K_{0.5}$</td>
</tr>
<tr>
<td>l-Malate (0.25–100 mM; pH 7.4) with NAD$^+$</td>
<td>18.86 ± 0.52$^*$</td>
</tr>
<tr>
<td>l-Malate (0.25–100 mM; pH 7.4) with NADP$^+$</td>
<td>40.38 ± 0.51$^*$</td>
</tr>
<tr>
<td>Anaplerotic reaction (pyruvate carboxylation)</td>
<td>91.98 ± 6.73</td>
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</tbody>
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$^*$ Kinetics for these reactions are sigmoidal and the reported values are $K_{0.5}$ values.

**Figure 5.** PPDK deficiency sensitizes Mtb to propionate toxicity, which is rescued by the addition of vitamin B12. A, Δppdk growth is inhibited in the presence of propionate. Bacterial growth measured by absorbance was determined for WT (solid blue), Δpck (solid purple), Δppdk (solid yellow), and Δppdkppdk (dashed yellow) grown for 14 days in 7H9 medium containing glycerol and 20 mM sodium propionate. B, propionate toxicity in Δppdk is rescued by the addition of 10 μg ml$^{-1}$ of vitamin B12 (VB12). Bacterial growth measured by absorbance was determined after 14 days. Error bars are representative of S.E. of 3–6 independent biological replicates. **, $p < 0.01$; ***, $p < 0.001$ relative to the WT control.

ppdk (Fig. 5A) demonstrating that PPDK plays a role in detoxifying propionyl-CoA. In contrast Δpck was not intoxicated by either cholesterol or propionyl-CoA (Table S2, Fig. 5A). In fact deletion of pck enhanced the growth of Mtb in the presence of propionate as compared with the WT strain (Fig. 5A).

*Mtb* has two pathways for metabolizing propionyl-CoA, via the methyl citrate cycle to yield pyruvate and succinate or in the presence of sufficient vitamin B12 the methyl malonyl pathway to yield succinyl-CoA. Activation of the methyl malonyl pathway with vitamin B12 restored growth of Δppdk in propionate (Fig. 5B) suggesting that PPDK provides a link between the methyl citrate cycle-derived pyruvate and the rest of metabolism.

To further explore the intracellular metabolic profile of this mutant we performed $^{13}$C isotopologue experiments with prelabeled THP-1 macrophages. THP-1 macrophages were passaged in $[^{13}$C]glucose/RPMI as previously described (7). These were infected with WT and Δppdk for 48 h. We selected this time point as the Δppdk mutant showed no detectable growth phenotype at this time so fluxes could be compared with WT (Fig. 2). Also, the labeling profile of amino acids is at an isotopic steady state by 48 h (7). These data were analyzed manually using the results from our previous flux analysis as a template (7). We focused the analysis on amino acids with $^{13}$C profiles, which were significantly different from the WT to identify major defects in metabolism consequent to enzyme deletion (Table S4, Fig. 6).

In the absence of PPDK there was a block in gluconeogenic flow (Table S4, Fig. 6). $^{13}$C enrichment occurred only in amino acids derived from metabolites occurring below pyruvate but not in those amino acids derived from glycolytic intermediates (Fig. 6, Table S4). These data show that during intracellular growth PPDK provides an alternative to PCK as a gateway into gluconeogenesis and that both of these routes are required for the intracellular survival of Mtb.

**Discussion**

A number of studies highlight the importance of individual enzymes of glycolysis, gluconeogenesis, and the TCA cycle to the virulence strategy of Mtb. These pathways are interconnected by four enzymes at a metabolic cross-roads known as the ANA node (9). Here we demonstrate that although there is some functional overlap, each of these enzymes fulfills a unique metabolic function during intracellular growth. In addition to its canonical role in gluconeogenesis and anaplerosis we reveal that the ANA node has unexpected roles in lipid biosynthesis and detoxifying cholesterol and propionate.

We previously demonstrated that Mtb is able to fix carbon from CO$_2$ both in vitro (15) and also when growing intracellularly (7). We show here that Mtb has three potential routes for the replenishment of C4 intermediates catalyzed by PCK, PCA, or MEZ and that disruption of two of these routes severely compromises the intracellular survival of Mtb demonstrating that CO$_2$ fixation plays a fundamental role in Mtb’s survival in its human host cell. At ambient CO$_2$ levels in vitro PCA was the dominant anaplerotic enzyme required for growth on pyruvate but at higher CO$_2$ levels this enzyme was redundant. However, deletion of both PCA and PCK from Mtb led to severe intracellular growth defects.
PCA enzymes are among the most efficient carboxylating enzymes, whereas all others including MEZ and PCK have poor activity under ambient CO₂ concentrations (21). It was previously shown by us and others that PCK can also function as a carboxylase (7, 16). Here, functional analysis of \( \textit{Mtb} \) MEZ confirms that this enzyme can also function as a carboxylating enzyme. However, for MEZ to contribute to TCA cycle replenishment, pyruvate would need to accumulate to high intracellular concentrations and therefore MEZ is likely to play a subordinate role when alternative anaplerotic routes (PCA and PCK) are operational. Moreover MEZ was unable to compensate for the loss of both PCA and PCK in the complex intracellular environment.

Malic enzymes have been studied extensively in eukaryotes. However, the role of this enzyme in pathogenic bacteria is not well understood. Potential roles include provision of: 1) pyruvate during gluconeogenesis; 2) carbon during anaplerosis; and 3) reducing power for fatty acid synthesis (22). We confirmed that \( \textit{Mtb} \) MEZ can function anaplerotically as a carboxylase although this enzyme preferentially catalyzes the oxidative decarboxylation of malate to pyruvate. Deletion of \( \textit{mez} \) had no effect on metabolism of glycolytic or gluconeogenic substrates. However, we detected alterations in the colonial morphology, cell wall lipid composition, and an intracellular invasion phenotype in MEZ-deficient \( \textit{Mtb} \). Interestingly the excess of free mycolates detected in the mutant has also been observed in mycobacterial biofilms where it is associated with the activity of an esterase (23). Although this enzyme has a preference for NAD, \( \textit{Mtb} \) MEZ is one of a small number of malic enzymes that have duel cofactor specificity and can therefore produce both NADH and NADPH as reductants. The dogma is that the dehydrogenase reactions of the oxidative pentose phosphate pathway are the major providers of reducing power in bacteria. However, the importance of alternative NADPH-generating

Figure 6. Deletion of PPDK disrupts gluconeogenesis of intracellular \( \textit{Mtb} \). Shown is \(^{13}\text{C}\) isopomer incorporation into proteogenic amino acids isolated from intracellular WT and \( \Delta \text{ppdk} \) after 48 h infection of pre-labeled (with [U-\(^{13}\text{C}_6\)]glucose) THP-1 macrophages superimposed on a metabolic network of central metabolism with the positioning of each chart indicating the source of the carbon backbone of each amino acid. Only the amino acids that had significant changes as compared with WT are displayed. \textit{Error bars} indicate S.D. of 3 to 4 samples from independent macrophage infections.
The anaplerotic node of Mtb

reactions has recently become evident (24). Malic enzymes are associated with a role in controlling lipid metabolism through the provision of NADPH in eukaryotes (22) and we hypothesize that this is also the case for Mtb, which is unusual in requiring both NADH and NADPH as reductants to fuel its fatty acid synthase systems, FASI and -II. Malic enzyme may be particularly important when Mtb is dieting on carbon sources, which have low flux through the pentose phosphate pathway. We are currently exploring this hypothesis further.

During intracellular growth Mtb is reliant on gluconeogenesis to metabolize a mixture of substrates including amino acids (7), fatty acids and cholesterol (17), and mutants lacking genes involved in gluconeogenesis fail to establish infections in macrophages and murine models of TB (4, 6). Gluconeogenesis is essential for the pathogen to make amino acids (serine, histidine, phenylalanine, and tyrosine) in addition to sugars such as trehalose, which are essential for cell wall biosynthesis as well as puridines to make DNA and RNA. The inability of the pck deletion mutant to grow in vitro on any gluconeogenic substrate shown here and by others (4) indicated that PCK cannot be functionally replaced by PPDK in vitro suggesting that this enzyme was not operative in Mtb (25). Here we show that this is not the case and that PPDK is essential for intracellular survival. Indeed 13C isotopomer analysis demonstrated that this enzyme is in fact an essential gateway into gluconeogenesis during intracellular growth. This work further confirms that gluconeogenesis is critical for intracellular growth of Mtb and reveals that PPDK is essential in this process.

The metabolism of host cholesterol has an essential role in TB infection (17, 26, 27) and therefore cholesterol metabolism has been highlighted as a drug target (28, 29). Here we have shown that PPDK has an essential role in the metabolism of cholesterol. We further demonstrated that strains lacking PPDK fail to grow if cholesterol is present in the medium. This form of intoxication has been described for other mutants (20, 26, 30) where it is associated with the inability to assimilate the cholesterol breakdown product propionyl-CoA and we confirmed that this is also the case here.

Propionyl-CoA metabolism is central to the adaptation of Mtb to growth on cholesterol (26). Mtb has three strategies for metabolizing propionyl-CoA via: 1) the methyl citrate cycle to produce pyruvate and succinate; 2) the methyl malonyl pathway leading to succinyl-CoA production; or 3) incorporation into cell wall lipids (19). The growth of Δppdk was inhibited by propionate even in the presence of an alternative carbon source demonstrating that propionate, or its by-products, were intoxicating this mutant. Activation of the methyl malonyl pathway with vitamin B12 rescued this phenotype, indicating that PPDK provides a link between the methyl citrate cycle and the rest of metabolism. Propionyl-CoA toxicity is not fully understood in bacteria including Mtb but this metabolite has been shown to be an inhibitor of several key metabolic enzymes including pyruvate dehydrogenase (PDH) (31, 32). The end product of the methyl citrate cycle is pyruvate, which could be channeled through PPDK to circumvent the requirement for PDH. Deletion of the alternative gluconeogenic route driven by PCK actually mitigates propionyl-CoA toxicity indicating that PPDK provides a uniquely essential gluconeogenic route for metabolizing propionyl-CoA-derived metabolites.

We posit that during intracellular growth when Mtb is co-metabolizing a mixture of gluconeogenic carbon substrates (6, 7), having two gluconeogenic gateways provides Mtb with a selective advantage. PPDK provides more efficient recovery of carbon from substrates that are metabolized via pyruvate such as propionyl-CoA. Through PCK a minimum of 2 mol of pyruvate are required to form 1 mol of PEP via PDH and the glyoxylate- or tricarboxylic acid cycle, whereas the molar ratio is 1:1 using PPDK. However, an additional ATP is necessary to regenerate ADP from AMP when using PPDK and therefore PCK provides an energy efficient route to make sugars from lipids. Utilizing both routes provides Mtb as an effective strategy to minimize propionyl-CoA toxicity and maximize ATP production. The absence of PPDK orthologues in mammals should facilitate the development of gluconeogenesis inhibitors as new drugs for the treatment of tuberculosis.

In summary we show that the ANA node acts as a gateway into gluconeogenesis and anaerobiosis in addition to non-canonical roles in cholesterol detoxification and lipogenesis and is essential for the intracellular life cycle of Mtb. Deregulating this node offers an attractive alternative to conventional antimicrobial chemotherapy.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli strain DH5α was grown in solid or liquid Luria-Bertani (LB) media. Frozen stocks of Mtb were cultivated using Middlebrook 7H11 agar containing 5% (v/v) oleic acid/albumin/dextrose/catalase (OADC) enrichment medium supplement (BD) and 0.5% (v/v) glycerol or Middlebrook 7H9 broth containing 0.2% (v/v) glycerol, 0.2% (v/v) Tween 80, and 5% (v/v) ADC. When selection was required, kanamycin at 20 μg ml⁻¹, hygromycin at 50 μg ml⁻¹, X-gal at 50 μg ml⁻¹, and zeomycin at 25 μg ml⁻¹ were added to the culture medium.

For the sole carbon growth experiments cultures were grown until late exponential phase (A₆₀₀ = 1.0) in complete 7H9, washed once with PBS, and plated in triplicate onto Roisin’s minimal agar (33) containing sole carbon sources, dual carbon sources, and multiple carbon sources. For consistency the carbon source(s) were added to attain an equivalent carbon concentration of 109 mM with the exception of acetate, which was added at 12 mM as higher amounts were toxic to Mtb. Duplicate plates for each substrate were incubated for up to 12 weeks. To test for any mutations that may have given false results any positives after 6 weeks were independently tested at least twice more.

Growth curves were performed in 7H9 or Roisin’s minimal media. For the propionyl-CoA toxicity assays strains were grown in 7H9 media containing 0.2% (v/v) glycerol, 0.2% (v/v) Tyloxoprol, and 5% (v/v) ADC and 20 mM sodium propionate. Vitamin B12 (10 μg ml⁻¹) was added when indicated. Cultures were inoculated with 1% (v/v) of washed late log phase culture (A₆₀₀ = 1.0). Cell growth was monitored daily.
The anaplerotic node of Mtb

Genetic manipulation

The ppdk mutant was constructed using the strategy described by Stewart et al. (34). Approximately 1-kb regions flanking ppdk were PCR amplified and cloned on either side of the hygromycin cassette of the suicide vector pG5, which is a pSMT100 plasmid carrying a kanamycin resistance gene in addition to the sacB counter-selectable marker. The resulting plasmid was electroporated into Mtb H37Rv as described previously (35). Double crossovers were selected for on 7H11 agar supplemented with kanamycin, hygromycin, and X-gal. The pca gene was disrupted by single step homologous recombination using the specialized phage transduction protocol (36). The mycobacterial recombineering system was used for the construction of the single pck and the double pca–pck mutants as described (37). Briefly, the allelic exchange substrates were constructed by PCR amplification of ~500 bp corresponding to regions upstream and downstream of the gene and subsequently inserted into pNCMT so that they flank an antibiotic resistance gene, hygromycin was used as the antibiotic marker for Δpck, and zeomycin was used to generate the ΔpcaΔpck mutant. The allelic exchange substrates were then electroporated into Mtb strains expressing phage recombinases. To restore the phenotypes the WT genes were reintroduced into the mutant strains using suicide vectors that insert into the attB site of Mtb. Using operon prediction software available online, pck was predicted not to be in an operon. ppdk was predicted to be the first gene in its operon therefore these genes and 500 bp upstream of the CDS were reintroduced back into Mtb using the plasmid pMV306. pca was predicted to be within an operon, and therefore this gene was cloned downstream of the hsp60 promoter and reintroduced into the genome using the suicide vector pMV361 PCR. Whole genome sequencing was performed to verify the expected genotypes for all mutants.

Expression and purification of recombinant MEZ

MEZ was cloned into pNIC28-BSA4, a vector for inducible expression of His-tagged MEZ in E. coli using the primers 5’-TACTTCAATCCATGAGGAGCCGCGGT and 3’-TATCCACCTTTACTGTTAGTCATATGCCGGGAG to generate the pNIC_MEZ overexpression plasmid. The pNIC_MEZ overexpression plasmid was confirmed by Sanger sequencing and contained a 1-kb fragment of the pca–pck operon. The resulting plasmid was subsequently transformed into the Tuner cells (Novagen) for overexpression of the MEZ gene. The pNIC_MEZ overexpression plasmid was co-transformed into E. coli Tuner cells (Novagen) for overexpression of the MEZ gene in Magic Media™ (ThermoFisher Scientific) containing 50 µg ml⁻¹ of kanamycin at 37 °C overnight. The supernatant containing soluble protein was isolated using a nickel affinity-based His-trap column (GE Healthcare). Unbound protein was removed by washing with 15 CV of lysis buffer (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1 mM β-mercaptoethanol, 5 mM MgCl₂, 0.5 mM CaCl₂, 2% glycerol, 0.1% Triton X-100, 25 mMimidazole) before the protein was eluted in 45 mM Tris, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM CaCl₂, 4% glycerol, 0.1% Triton X-100, 250 mM imidazole. The protein was dialyzed against 50 mMTris, pH 7.6, 100 mM NaCl, 5 mM MgCl₂, 0.5 mM CaCl₂, 4% glycerol for 4 h at 4 °C, and then stored at −80 °C.

MEZ enzyme activity was measured at 30 °C by spectrophotometrically measuring the NAD(P)H formation or utilization at 340 nm. Assaying in the direction of pyruvate formation the reaction mixture contained: 100 mM Tris, pH 7.4, 100 mM malate, 0.5 mM MnCl₂, 1.6 mM NAD(P). Each reaction mixture used to assay for pyruvate reductive carboxylating activity contained 100 mM Tris, pH 6.0, 50 mM pyruvate, 200 mM KHCO₃, 0.5 mM MnCl₂, and 0.6 mM NAD(P)H.

Cultivation of human THP-1 macrophages

The THP-1 human monocyctic cell line was obtained from ATCC TIB-202. Cells were grown in RPMI 1640 medium supplemented with 0.2% glucose, 0.2% sodium bicarbonate, and 10% heat inactivated fetal calf serum (FCS) (Sigma).

Intracellular bacterial growth assays

THP-1 cells were differentiated with 50 nM phorbol 12-myristate 13-acetate for 72 h at 37 °C, 5% CO₂, and 95% humidity. Cells were washed with PBS supplemented with 0.49 mM Mg²⁺ and 0.68 mM Ca²⁺ (PBS⁺) and 1% FCS. Macrophages were infected with different strains of Mtb at a multiplicity of infection of 5. After a 4-h incubation the macrophages were washed and incubated for 48 h before the mammalian and bacterial fractions were separated and used as a probe for the analysis of 13C labeling of amino acids in host macrophages and intracellular Mtb respectively.

13C isotopologue profiling of intracellular Mtb

13C isotopologue profiling was performed as described previously (15). Briefly labeled or unlabeled THP-1 macrophages were infected with different strains of Mtb at a multiplicity of infection of 5. After a 4-h incubation the macrophages were washed and incubated for 48 h before the mammalian and bacterial fractions were separated and used as a probe for the analysis of 13C labeling of amino acids in host macrophages and intracellular Mtb respectively.

13C biomass hydrolysate and preparation of amino acid derivatives

Amino acid derivatives were prepared from bacterial and host cell fractions as previously described (15). These were corrected for the natural abundance of all stable isotopes.

Extraction and analysis of lipids from mycobacterial strains

Extraction of polar, apolar lipids, and MAMEs from the conditional mutant, and subsequent TLC analysis was carried out using protocols described in Ref. 18. Dry weights of cell pellets were used as a measure to equalize loading on TLC plates using solvent systems A–D for apolar lipids and systems D and E for polar lipids. The GC-free induction decay analysis of FAMES was performed on a Shimadzu GC-2010. The FAMES separation was achieved using a DB-225 column (30 m × 0.25-mm inner diameter, 0.25 µm film thickness, J&W Scientific) with a column flow of 1.26 ml/min (helium at a constant pressure of 100 kilopascal). Injector and detector temperatures were 50 °C. The injection volume was 5 µl. The column temperature was programmed initially at 50 °C for 2 min, increased at the rate of 4 °C/min to 220 °C, and then maintained at 220 °C for a further 30.5 min. The total program time was 75 min.
The anaplerotic node of Mtb

Mycobacteria which requires isocitrate lyase and carbon dioxide fixation. 
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The anaplerotic node of Mtb

The anaplerotic node is essential for the intracellular survival of Mycobacterium tuberculosis


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