Central Role of Pyruvate Kinase in Carbon Co-catabolism of Mycobacterium tuberculosis*

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Tahel Noy‡, Olivia Vergnolle‡, Travis E. Hartman‡, Kyu Y. Rhee‡, William R. Jacobs, Jr.§,¶, Michael Berney†, and John S. Blanchard‡
From the Departments of ‡Biochemistry and ¶Microbiology and Immunology, Albert Einstein College of Medicine, New York, New York 10461, the ‡Department of Microbiology and Immunology, Howard Hughes Medical Institute at Albert Einstein College of Medicine, New York, New York 10461, and the ‡Department of Microbiology and Immunology, Weill Cornell Medical College, New York, New York 10021

Mycobacterium tuberculosis (Mtb) displays a high degree of metabolic plasticity to adapt to challenging host environments. Genetic evidence suggests that Mtb relies mainly on fatty acid catabolism in the host. However, Mtb also maintains a functional glycolytic pathway and its role in the cellular metabolism of Mtb has yet to be understood. Pyruvate kinase catalyzes the last and rate-limiting step in glycolysis and the Mtb genome harbors one putative pyruvate kinase (pykA, Rv1617). Here we show that pykA encodes an active pyruvate kinase that is allosterically activated by glucose 6-phosphate (Glc-6-P) and adenosine monophosphate (AMP). Deletion of pykA prevents Mtb growth in the presence of fermentable carbon sources and has a cidal effect in the presence of glucose that correlates with elevated levels of the toxic catabolite methylglyoxal. Growth attenuation was also observed in media containing a combination of short chain fatty acids and glucose and surprisingly, in media containing odd and even chain fatty acids alone. Untargeted high sensitivity metabolomics revealed that inactivation of pyruvate kinase leads to accumulation of phosphoenolpyruvate (P-enolpyruvate), citrate, and aconitate, which was consistent with allosteric inhibition of isocitrate dehydrogenase by P-enolpyruvate. This metabolic block could be relieved by addition of the α-ketoglutarate precursor glutamate. Taken together, our study identifies an essential role of pyruvate kinase in preventing metabolic block during carbon co-catabolism in Mtb.

Mycobacterium tuberculosis (Mtb) pathogenesis has been studied for decades, however, our knowledge concerning the metabolism and physiology of the bacterium during host infection is still limited (1–4). Specifically, we lack understanding of nutrient availability in the host microenvironments during the different phases of infection and consequently, which nutrients (e.g. carbon and nitrogen sources) are used by the bacterium for growth and maintenance of replicative and non-replicative states (5). In vitro evidence suggests that Mtb does not utilize carbon catabolite repression, a regulatory mechanism that allows bacteria and single-cell eukaryotes to gain growth advantage through prioritized metabolism of one carbon source over the other (6), but rather co-catabolizes multiple carbon sources at once (4). Several lines of evidence suggest that Mtb relies mainly on fatty acid metabolism in the non-replicative state within the host (1, 7–12). During growth on fatty acids Mtb bypasses the carbon dioxide releasing steps of the TCA cycle by running the glyoxylate shunt to conserve carbon (4). Nevertheless, Mtb maintains a functional and intact glycolytic pathway, suggesting that glycolysis plays a role under certain in vivo conditions. Recent studies with the adenosine triphosphate (ATP) synthase inhibitor, Bedaquiline, showed a delayed cidal effect when Mtb was grown on fermentable carbon sources (13), suggesting that Mtb can produce ATP through substrate level phosphorylation when oxidative-phosphorylation is limited. However, studies in mice with Mtb strains containing knockouts of glycolytic enzymes did not result in strong attenuation, thus leaving the role of glucose metabolism in the virulence and physiology of Mtb unclear (14, 15).

Pyruvate kinase (PK) catalyzes the final step in glycolysis in which phosphoenolpyruvate (P-enolpyruvate) and adenosine diphosphate (ADP) are converted to pyruvate and ATP. The product, pyruvate, is then used to prime the tricarboxylic acid (TCA) cycle with carbon metabolites. PK is one of the rate-limiting steps of glycolysis, thus potentially controlling the flux through and out of glycolysis (16). In many organisms, PK is a crucial point for regulating the switch between glycolysis and gluconeogenesis, serving to prevent a futile cycle between glycolysis and gluconeogenesis (17–22). In humans, during tumor development, proliferating malignant cells up-regulate the expression of a less active PK, PKM2, which leads to the accumulation of upstream glycolytic metabolites that serve as precursors for the synthesis of phospholipids and nucleotides needed for the replicating cells (23). In bacteria, inhibitors designed against PK from Staphylococcus aureus showed bacteriociidal effects against methicillin-resistant S. aureus (MRSA) strains and a wide range of both Gram-positive and Gram-negative bacteria, thus suggesting PK as a potential antibacterial drug target (24).

In most studied organisms, PK activity is regulated by the upper glycolysis metabolite, fructose 1,6-bisphosphate (25–27).
Through this feed-forward activation mechanism of PK, the rates of upper glycolysis, the ATP investing steps, and lower glycolysis, ATP producing steps, are balanced (28). This balance allows for the net production of 2 ATP and 1 nicotinamide adenine dinucleotide (NADH) molecules per molecule of glucose metabolized during glycolysis. The feed-forward activation of PK prevents the accumulation of intermediate glycolytic metabolites, which can undergo unfavorable, non-enzymatic side reactions, resulting in the production of toxic metabolites such as methylglyoxal (MG) (29, 30).

Despite its importance for the metabolism of different organisms, PK had been thought to be dispensable for Mtb due to several observations. First, Mycobacterium bovis, the causative agent of bovine tuberculosis, contains an inactive PK enzyme, and thus lacks the ability to metabolize fermentable carbon sources (31). Second, gluconeogenesis through the combined reaction of pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK) was shown to be essential for survival of Mtb in mice, indicating that glycolytic substrates cannot be scavenged from this host (1).

Over the past few years, a limited number of studies have aimed at describing the role of PK in the physiology of Mtb. Kearing et al. (32) showed that complementing H37Rv with the M. bovis pykA gene yielded a strain whose colony morphology resembled that of M. bovis. Chavadi et al. (31) demonstrated that H37Rv ΔpykA up-regulated the expression of genes that are involved in fatty acid β-oxidation, suggesting that in the absence of PK, Mtb utilizes fatty acids as energy source. However, neither of these studies described the activity or potential role of PK in the global context of Mtb metabolism.

In this study we used biochemical and genetic approaches to define the role of PK and glycolysis in the metabolism of Mtb. We show that pykA encodes an active pyruvate kinase, which is subjected to allosteric regulation by the upstream glycolytic intermediate glucose 6-phosphate. We demonstrate that PK is essential for metabolism of glucose as a sole carbon source, for co-metabolism of glucose in combination with other carbon sources, and for odd-chain fatty acid metabolism. Finally, we present evidence that PK is important to control the levels of MG and activity of isocitrate dehydrogenase.

**Experimental Procedures**

**Materials—**All chemicals were purchased from Sigma.

**Expression and Purification of Mtb PK—**The pykA gene (Rv1617) was PCR amplified from Mtb H37Rv genomic DNA using primers PK_Rv_XhoI (GAACTCGAGTCAGACGTCAAACAG) and PK_Fw_NdeI (GGGTTAATTCGCGGGAAAATCGC) and PK_Rv_XhoI (GAACTCGAGTCAGACGTCAACACAG) and cloned into pET28 plasmid and transformed into T7 express Escherichia coli cells (Invitrogen). A single colony was selected, grown in 25 ml of LB medium supplemented with 30 μg/ml of kanamycin. These cells were used to inoculate 6 liters of the same media. The cells were grown to mid-log phase (A600 = 0.6) at 37 °C, induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and cultured for 18 h at 18 °C. The cells were harvested by centrifugation and the pellet was resuspended in buffer containing 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl (buffer A). The cells were lysed in an EmulsiFlex-C3 homogenizer (Avestin) and centrifuged for 1 h at 38,000 × g. The supernatant was loaded onto a nickel-nitrilotriacetic acid column (Qiagen) pre-equilibrated with buffer A. The unbound proteins were eluted with 5 column volumes of 10 mM imidazole in buffer A, and eluted with a 20 column volume linear imidazole gradient, from 10 to 250 mM imidazole. The N-terminal His6 tag was removed by overnight thrombin cleavage in 100 mM HEPES, pH 8.0, containing 100 mM KCl and 10 mM CaCl2 followed by size exclusion chromatography using Supedex200 (GE Healthcare). Fractions containing pure PK were identified by SDS-PAGE followed by Coomassie Blue staining. Fractions were pooled and dialyzed against 100 mM HEPES, pH 7.5, containing 100 mM KCl, followed by concentration with an Amicon concentrator (Millipore) with a 30-kDa cutoff.

**Enzyme Activity Assay—**Initial velocities of the PK reaction were assayed spectrophotometrically by coupling the formation of pyruvate from P-enolpyruvate and ADP to the reaction of lactate dehydrogenase following the decrease in absorbance of NADH at 340 nm (ε340 = 6220 M⁻¹ cm⁻¹). All reactions were performed at 25 °C using a Shimadzu UV-2450 spectrophotometer. A typical reaction mixture contained 100 mM HEPES, pH 7.5, 100 mM KCl, 20 mM MgCl2, 0.1 mM NADH, pyruvate kinase, 6 units of lactate dehydrogenase, PK at a final concentration of 25 nM, and variable concentrations of the substrates: P-enolpyruvate and ADP. The reaction mixtures were incubated for 1 min at room temperature and initiated by the addition of P-enolpyruvate. Reactions were followed for 3–5 min and reaction velocities were calculated assuming 1 molecule NADH was oxidized for each pyruvate molecule formed. The activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured spectrophotometrically by monitoring the conversion of NAD⁺ to NADH at 340 nm. Reactions were conducted in 100 mM HEPES, pH 8, including substrates NAD⁺, Na₂AsO₄, and varying concentrations of Glc-3-P in a total volume of 0.5 ml. The activity of ICDH was measured by following the conversion of oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺) to NADPH at 340 nm. A typical reaction was carried out in 100 mM HEPES, pH 7.5, containing 5 mM MnCl₂, and varying amounts of isocitrate and fixed concentrations of NADP⁺, in a final reaction volume of 0.5 ml.

**Data Analysis—**The kinetic parameters for ADP were determined by fitting the initial velocity data for each concentration to Equation 1 using GraphPad 6.

\[
v = \frac{(V_A)}{(K + A)}
\]

(Eq. 1)

The kinetic parameters for P-enolpyruvate were determined by fitting the initial velocities data for each concentration to Equation 2 using GraphPad 6.

\[
v = \frac{VA^H}{(K_{0.5} + A^H)}
\]

(Eq. 2)

Where \( v \) is velocity, \( V \) is maximal velocity, \( A \) is substrate concentration, \( K_{0.5} \) is \( K_{\text{half}} \), \( K \) is the Michaelis constant, and \( H \) is the hill constant.

**Bacterial Strains and Growth Conditions—**The bacterial strains used in this study are listed in Table 1. Mtb mc²6230 and...
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TABLE 1

| Strain number | *M. tuberculosis* parent | Genotype | How constructed | Source |
|---------------|--------------------------|----------|----------------|--------|
| mc2           | H37Rv                    | Wild type | Ref 57 <xref> |        |
| mc2630        | H37Rv                    | ΔapmCD   | Specialized transduction of mc2630 with pHAΔpykA | This work |
| mc7887        | H37Rv                    | ΔapmCDΔpykA::hyg | Transformation of mc7887 with pMV306::pykA | This work |
| mc7888        | H37Rv                    | ΔapmCDΔpykA::hyg attB35::pYUB1951 | Transformation of mc7887 with pMV306::pykA | This work |
| mc2600        | CDC1551                  | Wild type | Specialized transduction of mc2600 with pHAΔpykA | This work |
| mc7490        | CDC1551                  | ΔpykA::hyg | Transformation of mc7490 with pMV306::pykA | This work |

CDC1551 were grown in modified Hartmans de Bondt (HdB) minimal medium (33) supplemented with the relevant carbon sources. When fatty acids were used as carbon sources (propionate, butyrate) media were supplemented with 10 μg/ml of vitamin B12. Selective media plates consisted of Middlebrook 7H9 supplemented with 5 g/liter of bovine serum albumin (BSA), 0.05 g/liter of oleic acid, 0.004 g/liter of catalase, 0.85 g/liter of NaCl, 40 mM pyruvate, 50 μg/ml of pantothenic acid, and either 75 μg/ml of hygromycin or 20 μg/ml of kanamycin. The gene *pykA* (Rv1617) was deleted by specialized transduction as described previously (34) and confirmed by PCR using primers Rv1617R (CGTCCAACTCGTACTTCTC), Rv1617L (GCTGATATTGACCGAAAAG) and a universal uptag (GATGCTACTGAGGTCTTCT). ΔpykA was complemented with the plasmid pMV306 (35) harboring a copy of *pykA* and 500 bp of upstream sequence containing the native promoter of the gene using primers Fw_PK_HindIII (AGCTAAGCTTGCTAGCACAC) and Rv_PK_XbaI (CTAGTCTA-GACTAGCTGTCATCTCCCG).

Metabolic Profiling—Cells were grown in HdB supplemented with either 10 mM glucose or 30 mM acetate. Metabolite extraction was performed as described previously (36). Briefly, 5 ml of cell culture was grown to A0.5 and quenched in 10 ml of 100% methanol at 4°C, spun down, and re-suspended in acetonitrile:methanol:water (2:2:1). Cells were lysed mechanically by using a bead-beater (MP Biomedicals), spun down, and metabolites were removed and filtered. Metabolite content was analyzed by UPLC-coupled mass spectrometry (Waters, Manchester, UK). Untargeted metabolomics was conducted as described (4).

Methylglyoxal Analysis—Cells were grown to mid-log phase in HdB supplemented with 5 mM glucose and either 15 mM acetate or 10 mM pyruvate. Metabolites were extracted and methylglyoxal was derivatized as previously described (37). Samples were analyzed by UPLC-coupled mass spectrometry.

Glucose Killing Assay—Cells were grown on HdB supplemented with 30 mM acetate until early log phase (A100 = 0.2). Cells were washed and inoculated into HdB media or HdB supplemented with either 10 mM glucose or 30 mM acetate to a final concentration of 1 × 10⁶ cells/ml. Cells were sampled on days 0 and 14 for cfu count and plated on agar plates containing Middlebrook 7H9 supplemented with 15 g/liter of agar, 5 g/liter of BSA, 0.05 g/liter of oleic acid, 0.004 g/liter of catalase, 0.85 g/liter of NaCl, and 40 mM pyruvate.

Mouse Experiments—Female SCID mice and female C57BL/6 mice (Jackson Laboratories) were infected via the aerosol route using a 1 × 10⁷ cfu/ml of mycobacterial suspension in PBS containing 0.05% tyloxapol and 0.04% antifoam. This yielded ~100 bacilli/lung as determined by a 24-h harvest of four mice per group. Subsequently, four mice from each group were sacrificed at days 1, 21, 56, and 112 to determine the bacterial burden in the lungs. SCID mice were kept for survival experiments. All mice infected with *Mtb* were maintained under appropriate conditions in an animal biosafety level 3 laboratory. Mouse protocols used in this work were approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

Ethics Statement—Mouse studies were performed in accordance to National Institutes of Health guidelines using recommendations in the Guide for the Care and Use of Laboratory Animals. The protocols used in this study were approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine (protocol 20120114).

Results

Pyruvate Kinase Activity Does Not Change in Acetate- or Glucose-fed Cultures—Pyruvate kinase has been shown to modulate flux through glycolysis in many different organisms, thus controlling the accumulation of glycolytic intermediates that are essential for cellular replication (22, 23, 25). As a glycolytic enzyme, the activity of PK is expected to be lower in the presence of a gluconeogenic carbon source to prevent a futile cycle between the glycolytic and gluconeogenic pathways (17–22). We tested if PK activity in *Mtb* is carbon source-dependent. *Mtb* was grown in HdB minimal medium containing acetate or glucose as the sole source of carbon. An enzymatically active cell lysate was prepared and the activity of different enzymes was tested. Surprisingly, PK activity was not altered in the acetate or the glucose-fed cell lysates (Table 2). Similarly, the activity of GAPDH and ICDH were unchanged in the acetate-fed and glucose-fed lysates (Table 2). This result demonstrates that PK is expressed and active regardless of the carbon source, suggesting that PK activity is necessary for carbon metabolism of fermentable and non-fermentable carbon sources.

*Mtb* PK Is Allosterically Activated by Glucose 6-Phosphate and AMP—Next we assessed if PK is allosterically regulated by intermediates of glycolysis. The most common metabolite that activates PK in other organisms is fructose 1,6-bisphosphate. Fructose 1,6-bisphosphate enhances the flux through glycolysis, and increases glucose uptake and catabolism (24, 25, 38). To measure the potential allosteric regulation of PK in Mtb we cloned, expressed, and purified PK<sub>Mtb</sub> in *E. coli*, and measured the kinetic parameters of the enzyme. When the enzyme was assayed at saturating concentrations of P-enolpyruvate and varying concentrations of ADP, it showed hyperbolic Michael-
lis-Menten kinetics (Fig. 1A). The data were fitted to Equation 1 and yielded $K_{\text{m}}^\text{ADP} = 0.81 \pm 0.10 \text{mm}$ and $V_{\text{max}} = 54 \pm 3 \text{s}^{-1}$. When assayed at saturating concentrations of ADP and varying concentrations of P-enolpyruvate (PEP) the enzyme exhibited sigmoidal kinetics (Fig. 1B). The data were fitted to Equation 2 and yielded $K_{\text{m}}^\text{PEP} = 1.0 \pm 0.1 \text{mm}$ and $V_{\text{max}} = 63 \pm 4 \text{s}^{-1}$ with $n^\text{m} = 2.04 \pm 0.4$.

The sigmoidal kinetics in the presence of increasing concentrations of P-enolpyruvate suggested that PK is subjected to allosteric regulation. However, neither fructose 1,6-bisphosphate nor ribose 5-phosphate activated the rate, nor changed the sigmoidal kinetics when P-enolpyruvate was the variable substrate (data not shown). Instead we found that AMP or Glc-6-P were able to activate PK 3.6- and 4.7-fold, respectively (Fig. 1C). This result suggests that PK influences the concentrations of upstream triose phosphates that can spontaneously degrade to MG, producing a toxic electrophilic species.

**PK Prevents Accumulation of Toxic Methylglyoxal in the Presence of Glucose**—Next we assessed glucose toxicity in the pykA mutant by measuring cell survival by colony forming units after 14 days exposure to either glucose or acetate, or in the absence of a carbon source. In the presence of glucose, the viability of the $\Delta pykA$ strain dropped 100-fold, but stayed constant when no carbon source was added (Fig. 3A). It has previously been suggested that one potential mechanism behind glucose toxicity relies on the accumulation of phosphorylated hexoses and trioses that give rise to the spontaneous side reactions that generate MG (3, 29). We compared the levels of MG in WT and mutant strains when grown on a medium containing glucose and acetate. MG levels were 3.5–4 times higher in the $\Delta pykA$ strain compared with the WT (Fig. 3B). This result suggests that PK influences the concentrations of upstream triose phosphates that can spontaneously degrade to MG, producing a toxic electrophilic species.

**Pyruvate Kinase Is Essential for Growth of Mtb on Propionate or Butyrate as Sole Carbon Sources**—Mtb relies on fatty acid catabolism during persistence (1, 7–12), hence we investigated if inactivation of PykA would affect the ability of Mtb to grow on short chain fatty acids. To do so, WT, $\Delta pykA$, and $\Delta pykA\text{comp}$ were grown in HdB medium supplemented with either butyrate (an even-chain fatty acid) or propionate (odd-chain fatty acids), or a combination of glucose with either of the fatty acids (Fig. 4). $\Delta pykA$ failed to grow in the combination of any short chain fatty acid with glucose (Fig. 4, A and B). Surprisingly, $\Delta pykA$ growth was also severely attenuated on HdB containing propionate (Fig. 4C) and failed to grow in the presence of butyrate (Figs. 4D), even though all fatty acid containing media were supplemented with 10 $\mu$g/ml of vitamin B12. Catabolism of odd-chain fatty acids results in the production of the toxic metabolite propionyl-CoA, which can be detoxified by the B12-dependent methylmalonyl pathway (39, 40). Hence, our results indicate that PK is essential for co-metabolism of glucose and short chain fatty acids, and essential for the metabolism of short chain fatty acids alone. This also suggested that inactivation of PK elicits a metabolic block beyond the accumulation of toxic glycolytic substrates. To address this hypothesis, we performed untargeted metabolomics on WT, $\Delta pykA$, and $\Delta pykA\text{comp}$ strains grown in minimal medium containing acetate or a combination of acetate and glucose. The $\Delta pykA$ strain accumulated P-enolpyruvate under both conditions compared with the WT strain, but P-enolpyruvate levels were substantially higher.

### TABLE 2

Kinetic parameters different enzymes in whole cell lysate of acetate- or glucose-fed cultures

| Enzyme      | Acetate ($K_{\text{m}}^{\text{ADP}}$) | Glucose ($K_{\text{m}}^{\text{ADP}}$) |
|-------------|--------------------------------------|--------------------------------------|
| PK          | 0.032 ± 0.001 mms                      | 0.031 ± 0.003 mss                     |
| ICDH        | 0.15 ± 0.01 mms                        | 0.13 ± 0.03 mss                       |
| GAPDH       | 0.89 ± 0.15 mms                        | 0.83 ± 0.2 mms                        |

* Activity of PK in the presence of 5 mms ADP and 0.02–0.8 mms PEP.
* Activity of GAPDH in the presence of 0.5 mms NAD+ and 0.2–4 mms Glc-3-P.
* Activity of ICDH in the presence of 1 mms NADH and 0.005–0.1 mms isocitrate.
when the ΔpykA strain was grown in the presence of glucose in the media (Fig. 5). Surprisingly, the ΔpykA strain also accumulated high levels of citrate and aconitate, which pointed to an inhibition of ICDH activity in the ΔpykA strain. In fact, it has been shown that P-enolpyruvate inhibits ICDH in E. coli (41) enabling feed-forward control of metabolic flux through the TCA cycle and glyoxylate shunt based on the activity of glycolysis. Therefore, we measured the activity of ICDH in Mtb whole cell lysates in the presence and absence of 10 mM P-enolpyruvate. We observed a 10-fold increase in the $K_m$ value of ICDH toward isocitrate in the presence of P-enolpyruvate (Fig. 6A), indicating that P-enolpyruvate inhibits ICDH in Mtb lysates. The metabolic block at ICDH depletes the cell of $\alpha$-ketoglutarate and the addition of this metabolite should rescue the pykA strain for growth on fatty acids. Because Mtb efficiently transports and converts glutamate to $\alpha$-ketoglutarate (42) we

![Figure 1. Activation of PK by allosteric effectors.](image)
added glutamate (0.5 g/liter) to the medium. Consistent with our hypothesis, glutamate rescued Mtb growth on propionate and butyrate (Fig. 6, B and C).

Pyruvate Kinase Is Not Essential for Mtb Infection in a Mouse Model—The inability of ΔpykA to efficiently co-catabolize fermentable and non-fermentable carbon sources as well as the absence of growth on short chain fatty acids pointed to a potential role of PK for survival in vivo. We tested Mtb ΔpykA for its ability to proliferate and survive in immunocompetent (C57BL/6) or immunocompromised (SCID) mice. Mice were infected with Mtb CDC1551, Mtb ΔpykA, and Mtb ΔpykAcomp via the aerosol route with ~100 bacteria per lungs to mimic the natural route of infection. Bacterial burdens in lungs from C57BL/6 mice were determined 1, 3, 8, and 16 weeks after infection (4 mice per group and time point), whereas SCID mice were left to determine mouse survival. No significant difference in bacterial lung burden between the different strains could be observed throughout the experiment (Fig. 7). However, SCID mice infected with the ΔpykA strain died significantly faster (10 days difference) than mice infected with WT or complemented strains (Fig. 7).
Discussion

The metabolic strategies used by Mtb to survive in the host are not well understood. Evidence exists that the diet of Mtb consist primarily of host lipids and fatty acids, however, this obligate intracellular pathogen has retained its ability to grow on sugars and readily co-catabolizes fermentable and non-fermentable carbon sources due to the absence of carbon catabolite repression (4). Our data demonstrate that regardless of the type of carbon source present in the media (glycolytic or gluconeogenic), the activities of PK, ICDH, and GAPDH remain unchanged, which argues that Mtb is constantly primed for carbon co-catabolism. However, this brings higher energetic costs for the cell due to a potential futile cycle between glycolytic and gluconeogenic enzymes and may explain why the growth rate of Mtb is so similar on fermentable and non-fermentable carbon sources. In the absence of carbon catabolite repression, other regulatory mechanisms, like allostery, are likely to play a more prominent role in the control of carbon metabolism in Mtb. Our results show that PK is allosterically regulated by Glc-6-P and AMP in a feed-forward fashion. As expected, Glc-6-P levels were higher in glucose grown cells than in acetate grown cells, which is consistent with previously published data (4). Rapid activation of glycolytic flux could be particularly important in situations where rapid reduction of sugar phosphate levels is needed to prevent accumulation of toxic intermediates such as methylglyoxal (29, 43), and to balance the ATP investing steps of upper glycolysis to ATP producing steps in lower glycolysis (44).

Genetic and metabolic studies indicate that Mtb is able to substitute the conversion of P-enolpyruvate to pyruvate by the reversible reaction of pyruvate phosphate dikinase (45) or via a combination of PEPCK and pyruvate carboxylase that can catalyze the conversion of P-enolpyruvate to oxaloacetate and then pyruvate (46). However, deletion of pykA rendered Mtb unable to grow on glucose or combinations of fatty acids and glucose, which argues that the proposed pathways could not

FIGURE 5. Metabolic profiling of WT (black), ΔpykA (red), and ΔpykAcomp (blue) growing in HdB solid media supplemented with acetate or a combination of acetate and glucose. n = 3, significance was tested by using t test. * represents p value < 0.005.
complement for the loss of PK under these conditions. This stands in contrast to the model bacterium *E. coli* where PK-deficient mutants can still grow on glucose due to the activities of PEPCK and pyruvate carboxylase albeit at a reduced growth rate (47, 48). A metabolic block at the level of PK in the presence of glucose is likely to increase the levels of the triose phosphates that convert to the toxic electrophile MG via a non-enzymatic reaction. Consistent with this mechanism, glucose was bactericidal for *Mtb*/*H9004 pykA* and correlated with the accumulation of MG.

Previous studies with a phosphofructokinase A knock-out strain of *Mtb* showed glucose toxicity under hypoxic but not aerobic conditions (15). The difference in phenotype may arise from the position of the two enzymes in the glycolytic pathway. As an upper glycolytic enzyme, deletion of phosphofructokinase A will result in accumulation of hexose phosphates that can be shunted through the pentose phosphate pathway to support nucleic acid and cell wall biosynthesis via a non-enzymatic reaction. Consistent with this mechanism, glucose was bactericidal for *Mtb* ΔpykA and correlated with the accumulation of MG.

The ΔpykA strain demonstrated an attenuated growth rate in the presence of glucose and additional carbon sources, suggesting that the lack of PK affects the ability of the bacteria to co-catabolize different carbon sources. However, the growth phenotype was not identical between the combinations of glucose with either acetate or pyruvate. The ΔpykA strain demonstrated substantial growth attenuation in the presence of glucose and acetate compared with glucose and pyruvate, indicating that pyruvate can partially rescue the growth phenotype. Pyruvate metabolism can proceed in *Mtb* via either lactate dehydrogenase, pyruvate dehydrogenase, or the reductive branch of the TCA cycle. Reduction of pyruvate via these pathways regenerates NAD⁺ molecules that were consumed in glycolysis and the TCA cycle, and helps in refueling these pathways. This cannot be achieved when the culture is supplemented with acetate, because it enters the TCA cycle through AcCoA, and requires NAD⁺ for its catabolism (51, 52).

The inability of the ΔpykA strain to grow on short chain fatty acids pointed to an additional metabolic block beyond the glucose toxicity effects described above. This observation could be explained by an accumulation of P-enolpyruvate that inhibits the activity of ICDH and leads to depletion of α-ketoglutarate,
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thus preventing an efficient flux through the TCA cycle. Reduced flux through the TCA cycle slows down anabolic reactions and energy production. Addition of the α-ketoglutarate precursor glutamate relieved this metabolic block, revealing an important role for glutamate in maintaining efficient flux through the TCA cycle during growth on fatty acids.

Despite the essential role of PK in carbon co-catabolism and fatty acid catabolism, we did not observe a difference in the mutants ability to replicate and persist in immunocompetent mice. However, the mutant was slightly more virulent than WT and the complemented strain in this experiment. Interestingly, M. bovis, a strain that naturally lacks pyruvate kinase activity is much more virulent than its Mtb relatives in mouse models (53). These results imply that pyruvate kinase might help in fine-tuning the in vivo growth rate of Mtb in mice. Knowing that ΔpykA cannot grow on short chain fatty acids in vitro, it is tempting to speculate that the in vivo diet contains substrates like acetate, lactate, and glutamate. In fact, mass spectrometric determination of the metabolome of Mtb-infected human macrophages and granulomatous tissue of guinea pigs show the presence of acetate, lactate, and glutamate (5, 54). Moreover, ^13^C-metabolic flux analysis of M. tuberculosis grown in THP-1 macrophages showed that glutamate is among the substrates preferentially taken up from the phagosome (55). Although our studies show that PK is not essential for Mtb pathogenesis in mice, it is important to note that mouse models do not perfectly mimic the human disease and that many enzymatic reactions, although non-essential for mouse infection, were shown to be essential to infect higher organisms (56).

Taken together, our data describe a central role of PK in actively growing Mtb cells and provides the first systematic characterization of PK in Mtb metabolism and pathogenesis. PK is crucial to facilitate co-catabolism of glycolytic and gluconeogenic substrates and is essential for the detoxification of sugar phosphates during glucose metabolism as well as for growth on short chain fatty acids. This argues that PK is needed to keep Mtb primed at all times to rapidly react to potential changes in carbon availability in its environment.

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