Two enzymes with redundant fructose bisphosphatase activity sustain gluconeogenesis and virulence in *Mycobacterium tuberculosis*

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The human pathogen *Mycobacterium tuberculosis* (*Mt*) likely utilizes host fatty acids as a carbon source during infection. Gluconeogenesis is essential for the conversion of fatty acids into biomass. A rate-limiting step in gluconeogenesis is the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate by a fructose bisphosphatase (FBPase). The *Mt* genome contains only one annotated FBPase gene, *glpX*. Here we show that, unexpectedly, an *Mt* mutant lacking *GLPX* grows on gluconeogenic carbon sources and has detectable FBPase activity. We demonstrate that the *Mt* genome encodes an alternative FBPase (*GPM2*, Rv3214) that can maintain gluconeogenesis in the absence of *GLPX*. Consequently, deletion of both *GLPX* and *GPM2* is required for disruption of gluconeogenesis and attenuation of *Mt* in a mouse model of infection. Our work affirms a role for gluconeogenesis in *Mt* virulence and reveals previously unidentified metabolic redundancy at the FBPase-catalysed reaction step of the pathway.
Mycobacterium tuberculosis (Mtb) is a resilient intracellular bacterium capable of infecting and surviving within host macrophages. Mtb’s ability to persist and ultimately establish a latent infection requires energy and biomass. Therefore, Mtb carbon metabolism is critical to the pathogen’s virulence and represents a new area for tuberculosis (TB) drug development. Our understanding of the enzymes and metabolic pathways that contribute to Mtb’s pathogenicity, however, remains incomplete.

The unique nature of Mtb carbon metabolism was reflected in an early observation that Mtb recovered from infected animals preferentially respired fatty acids instead of glycolytic substrates. A mounting body of evidence now suggests that fatty acids are a significant carbon source for Mtb during an infection. Following beta-oxidation, carbon from fatty acids can be readily directed towards the TCA cycle for energy production. Alternatively, carbon flux can be routed towards biomass production via gluconeogenesis, a pathway that generates glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), precursors for nucleotide and cell wall biosynthesis, respectively (Fig. 1). Thus, gluconeogenesis is critical to Mtb’s ability to convert fatty acids into biomass and is likely required for the pathogen to cause disease.

Early evidence for a role of gluconeogenesis in Mtb virulence comes from studies of isocitrate lyase ICL, an enzyme that operates in the glyoxylate shunt. Biomass production from fatty acids requires the glyoxylate shunt to bypass the oxidative branch of the TCA cycle, where carbon is lost as CO₂ and enable gluconeogenic carbon flow. Loss of ICL abolished growth of Mtb on fatty acids in vitro and led to early clearance of the pathogen from the lungs of infected mice. These phenotypes were attributed to a requirement for gluconeogenesis, as disruption of the glyoxylate shunt blocks the flow of carbon from fatty acids into this pathway. This interpretation, however, does not account for the fact that ICL also functions as a methylisocitrate lyase in the methylcitrate cycle. Besides inhibiting gluconeogenesis, loss of methylisocitrate lyase activity in ICL-deficient Mtb also perturbed Mtb’s intrabacterial pH and membrane potential. Given the pleiotropic effects of ICL deletion, it is unclear to what extent the ICL mutant’s in vivo growth and survival defects are caused by the interruption of gluconeogenesis.

Phosphoenolpyruvate carboxykinase (PEPCK), encoded in Mtb by pckA, catalyses the first committed step of gluconeogenesis, converting oxaloacetate into phosphoenolpyruvate. Loss of PEPCK in Mtb results in a block of gluconeogenesis, but the enzyme is dispensable for glycolysis. Similar to ICL-deficient Mtb, lacking PEPCK cannot utilize fatty acids to support in vitro growth and fails to establish and maintain infection in mouse lungs. Thus, the inability of PEPCK-deficient Mtb to survive in vivo is associated with a disruption of gluconeogenesis that renders the bacterium unable to utilize host fatty acids. However, PEPCK has been reported to also operate in the reverse direction, converting phosphoenolpyruvate into oxaloacetate to facilitate pyruvate distribution in metabolism under conditions of slowed growth. PEPCK’s anaplerotic activity brings into question whether the in vivo survival defect of PEPCK-deficient Mtb is solely due to disrupted gluconeogenesis.

Fructose bisphosphate aldolase (FBA) and triose phosphate isomerase (TPI) are also required for Mtb to grow on fatty acids in vitro, and mutants lacking these enzymes are attenuated in vivo. However, the reactions catalysed by these enzymes are bidirectional; loss of either FBA or TPI disrupts both gluconeogenesis and glycolysis. Thus, the extent to which loss of gluconeogenesis, as opposed to disruption of glycolysis, contributes to the in vivo survival defect of FBA and TPI mutants cannot be determined. The question of whether gluconeogenesis is required for Mtb virulence remains unanswered.

In this work, we sought to address the role of gluconeogenesis in Mtb virulence by studying fructose bisphosphatase (FBPase). FBPases catalyse the rate-limiting step of gluconeogenesis in which fructose-1,6-bisphosphate (F6P) is hydrolysed to yield F6P and inorganic phosphate. Five classes of FBPases have been defined based on primary sequence. Eukaryotes only encode the Type I FBPase, while all five types can be found among prokaryotes. Types I, II and III FBPases are expressed in bacteria, while the Type IV enzyme is found primarily in archaea. Type V FBPases are FBP aldolase/phosphatases that are associated with thermophilic prokaryotes from both archaea and bacteria. Unlike other steps in gluconeogenesis, the FBPase reaction is unidirectional and specific to this pathway. Thus, ablation of FBPase activity will only disrupt gluconeogenesis while leaving glycolysis unperturbed, allowing...
for a direct assessment of the specific role of gluconeogenesis in *Mtb* virulence.

GlpX (rv1099c) encodes the only annotated FBPase in the *Mtb* genome (Fig. 1). GLPX is classified as a Type II FBPase based on its homology to *Escherichia coli* FBPase of the same name and belongs to the metal-dependent/Li⁺-inhibited phosphomonoesterase protein family. The annotation of GLPX as an FBPase was validated by the demonstration that overexpression of this enzyme rescued growth and FBPase activity of an *E. coli* FBPase mutant on a gluconeogenic carbon source. Furthermore, recombinant GLPX has FBPase activity with a reported *Kₘ* of 44 μM and a *kₖₐₜ* of 1.6 s⁻¹. To study the role of FBPase and gluconeogenesis in *Mtb* virulence, we generated a *glpX* deletion mutant (*Δ*glpX). In agreement with previous TraSH and TnSeq analyses, we found that *glpX* is not essential for in vitro growth of *Mtb* on glycerol and fatty acids. We demonstrate that GPM2, a broad-specificity phosphatase, has FBPase activity that maintains *Mtb* gluconeogenesis in the absence of GLPX. Only deletion of both FBPases disrupted gluconeogenesis and rendered *Mtb* unable to establish infection, affirming the importance of this pathway to virulence.

**Results**

**GLPX is dispensable for growth on gluconeogenic carbon sources.** GlpX (rv1099c) is the only gene in the *Mtb* genome annotated to encode an FBPase. To study the role of FBPase in carbon metabolism, we generated ΔglpX, in which *glpX* was completely deleted and replaced with a hygromycin resistance cassette (Supplementary Fig. 1a). Southern blot analysis confirmed deletion of *glpX* (Supplementary Fig. 1b). Furthermore, GLPX protein was not detected from ΔglpX cell lysates by immunoblot and could be restored to levels observed in the wild-type (WT) strain by transformation of ΔglpX with a plasmid expressing *glpX* under the

Figure 2 | *Mtb* ΔglpX grows on gluconeogenic carbon sources and has detectable FBPase activity. (a) Growth of WT *Mtb* (black), ΔglpX (red) and complemented strain ΔglpX/C (grey) in Sauton’s minimal media containing no carbon source, 0.4% glucose, 0.1% glycerol or 0.1% butyrate. Data are representative of three independent experiments. (b) FBPase activity of WT *Mtb* (black), ΔglpX (red) and complemented strain ΔglpX/C (grey) cell lysates in the absence or presence of lithium chloride using 1.5 mM FBP or 12 mM FBP as substrate. Dashed line indicates limit of detection. Data are mean ± s.d. of three biological replicates.
control of a constitutive promoter (Supplementary Fig. 1c). WT and ΔglpX Mtb strains grew indistinguishably with the glycolytic carbon source glucose (Fig. 2a). Contrary to our expectations, ΔglpX grew just as well as WT Mtb with gluconeogenic carbon sources such as glycerol, acetate and butyrate (Fig. 2a and Supplementary Fig. 6). These results suggest that Mtb remains gluconeogenically competent in the absence of the FBPase GLPX.

Mtb may express a second FBPase sustaining gluconeogenesis when GLPX is absent or utilize a metabolic bypass pathway that enables gluconeogenesis without the use of an FBPase. A metabolic bypass pathway is expected to result in changes in the carbon flux via such a putative bypass. Loss of glpX, however, led to no significant changes in the levels of metabolites that participate in the carbon flux via such a putative bypass. Loss of glpX, however, led to no significant changes in the levels of metabolites that participate in gluconeogenesis/glycolysis (hexose-phosphate, pyruvate), the TCA cycle (α-ketoglutarate, oxaloacetate/aspartate) or the pentose phosphate pathway (sedoheptulose-phosphate) (Supplementary Fig. 2). While the lack of any significant changes in the glpX/(ΔglpX) metabolome does not support the existence of a metabolic bypass pathway, they demonstrate that gluconeogenesis in Mtb is not strictly dependent on GLPX. We thus hypothesized that Mtb may express a second FBPase that maintains gluconeogenesis in the absence of GLPX.

ΔglpX retains detectable, lithium-resistant FBPase activity. To address whether Mtb expresses a second FBPase, we measured FBPase activity in lysates of ΔglpX. In the presence of a substrate concentration that is saturating for GLPX (1.5 mM FBP), we were able to detect FBPase activity from WT Mtb cell lysate (Fig. 2b). We also detected FBPase activity from ΔglpX cell lysate that was reduced by 76% relative to that of WT Mtb and could be complemented by expressing glpX in the ΔglpX background. The remaining FBPase activity in ΔglpX supports the hypothesis that Mtb has a redundant enzyme with FBPase activity. It has been previously reported that Mtb’s GLPX is sensitive to inhibition by lithium (IC$_{90}$ Li$^+$ = 2.5 mM)$^{23}$. The mechanism of lithium inhibition is based on the ability of lithium ions to displace metal cofactors with a similar charge density like magnesium$^{21,27}$, which is an important metal cofactor for Mtb’s GLPX$^{23}$. In the presence of 10 mM LiCl, FBPase activity in WT was reduced to levels that were similar to those observed in ΔglpX. In contrast, FBPase activity in ΔglpX was not inhibited by the same LiCl treatment. Thus, WT Mtb FBPase activity is partially sensitive to lithium, and the lithium-resistant portion was retained in ΔglpX. Unlike GLPX, Mtb’s second FBPase appears to be lithium resistant. In the presence of a higher substrate concentration (12 mM FBP), we observed increased FBPase activity levels in both WT and ΔglpX, and there was no significant difference in the FBPase activity levels of these strains (Fig. 2b). Furthermore, lithium did not inhibit FBPase activity of these strains at this higher substrate concentration. The fact that FBPase activity in ΔglpX increased with increasing substrate concentration suggests that Mtb’s second FBPase has a lower affinity (that is, higher $K_M$) for FBP than GLPX. The residual FBPase activity detected in ΔglpX lysates could be inactivated by heating the lysate to $\geq 80$ $^\circ$C (data not shown). Taken together, these data support the existence of a second FBPase in Mtb that differs from GLPX in at least its substrate affinity and resistance to lithium inhibition.

Identification of the second FBPase in Mtb. To identify Mtb’s second FBPase, we took an unbiased biochemical approach and purified the FBPase activity from ΔglpX lysate using a series of chromatographic enrichments (Fig. 3a). The purification scheme involved four steps that separate proteins based on their charge, hydrophobicity and molecular weight. After each purification step, active fractions were identified using an FBPase activity assay and then pooled for further purification. Consistent with

**Figure 3 | Identification of the second FBPase by biochemical extraction.** (a) FBPase activity of the ΔglpX cell lysate was purified using a series of liquid chromatography techniques followed by SDS–PAGE of the final active fractions. (b,c) Silver-stained SDS–PAGE gel (b) and FBPase activity profile using 12 mM FBP as substrate (c) for the range of fractions from the final Mono Q anion exchange chromatography with detectable FBPase activity. Inactive fractions 1 and 15 were included in the SDS–PAGE analysis. A single band of $\sim$25 kDa (red arrow in b) correlated with the FBPase activity profile of the fractions. Peptide mass fingerprinting identified the major protein component of this band to be GPM2 (Rv3214, molecular weight = 21.95 kDa, 66.5% coverage, eight peptides).
our earlier observations, active fractions demonstrated robust FBPase activity in the presence of 12 mM FBP and were resistant to inhibition by lithium (tested in the presence of 1.5 mM FBP). After four purification steps, FBPase activity was enriched by at least 65-fold (Supplementary Table 1). We then visualized the remaining proteins in the final active fractions by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; Fig. 3b). We observed a single band of ~25 kDa whose intensity correlated with the FBPase activity profile of the active fractions (Fig. 3c).

Peptide mass fingerprinting identified the protein in this band as GPM2 (Rv3214, molecular weight = 21.95 kDa, 66.5% coverage, eight peptides, Supplementary Table 2), which has been reported to function as an acid phosphatase26.

GPM2 can dephosphorylate a variety of substrates including phosphorylated sugars like F6P25. FBP, however, had not been tested. While GPM2 had not been tested for sensitivity to lithium inhibition, it does not appear to require a metal cofactor, suggesting that it might be lithium resistant. While GPM2 does not belong to any of the five types of FBPases, it shares 31% identity with Saccharomyces cerevisiae YK23 (also known as SHB17), which has both FBPase28 and sedoheptulose bisphosphatase (SBPase) activity29. YK23 has a relatively high $K_m$ for FBP (0.5 mM) and also lacks a metal cofactor28. Based on this evidence, we hypothesized that GPM2 may serve as an FBPase in Mtb.

Validation of GPM2 as an FBPase. Expression of GLPX in an FBPase-deficient E. coli strain restored its ability to grow on gluconeogenic carbon sources22. We sought to perform a similar functional complementation test with GPM2 to validate it as an FBPase. We generated a Mycobacterium smegmatis (Msm) strain with a deletion of the glpX homologue, ΔglpX_Msm (Supplementary Fig. 3). While ΔglpX_Msm grew well on glucose, this strain demonstrated a partial growth defect on gluconeogenic carbon sources (Supplementary Fig. 4a). Similar to what was observed in Mtb, ΔglpX_Msm had reduced FBPase activity relative to WT and the residual activity was resistant to inhibition by lithium (Supplementary Fig. 4b). The partial growth defect of ΔglpX_Msm on gluconeogenic carbon sources could be fully complemented by overexpressing GLPX from Mtb (Supplementary Figs 4a and 3c). Overexpression of GLPX in ΔglpX_Msm also complemented the FBPase activity defect (Supplementary Fig. 4b). These results demonstrate that expression of an FBPase can functionally complement the growth and FBPase activity defects of ΔglpX_Msm. We reasoned that ΔglpX_Msm could be similarly used to test whether GPM2 can function as an FBPase in vivo. Indeed, overexpression of GPM2 in ΔglpX_Msm complemented the mutant’s partial growth defect on glycerol just as well as GLPX overexpression (Fig. 4a) and complemented the FBPase activity defect of ΔglpX_Msm (Fig. 4b). Moreover, the additional FBPase activity provided by GPM2 overexpression was resistant to inhibition by lithium.

To further validate GPM2 as an FBPase, we determined GPM2’s kinetic parameters with FBP as a substrate. Recombinant GPM2 demonstrated robust FBPase activity (Fig. 5a and Table 1). The enzyme’s FBPase activity followed allosteric sigmoidal kinetics with both a high $K_m$ (5.51 mM FBP) and a high $k_{cat}$ (1.87 × 10$^4$ s$^{-1}$) compared with GLPX. GPM2 FBPase activity was also resistant to inhibition by lithium (Fig. 5b). Thus, the enzymatic characteristics of GPM2 match the properties of the remaining FBPase activity observed in ΔglpX cell lysates. Both GLPX and GPM2 have specificity constants ($k_{cat}/K_m$) in the same range (2.3 × 10$^4$ s$^{-1}$ M$^{-1}$ and 3.4 × 10$^4$ s$^{-1}$ M$^{-1}$, respectively), suggesting that they function equally well as FBPases.

Was GPM2 responsible for the ability of ΔglpX to grow on gluconeogenic carbon sources? To address this question, we deleted gpm2 in the ΔglpX Mtb mutant background (Supplementary Fig. 5). While ΔglpXΔgpm2 grew as well as WT on glucose, it was unable to grow on any of the gluconeogenic carbon sources that we tested: glycerol, acetate and butyrate (Fig. 6a and Supplementary Fig. 6). Thus, the ability of ΔglpX to grow on gluconeogenic substrates was dependent on the expression of GPM2. The growth defect of ΔglpXΔgpm2 on gluconeogenic carbon sources was fully complemented by restoring expression of either GLPX or GPM2 (Fig. 6a and Supplementary Figs 5c,d and 6), demonstrating that Mtb requires expression of at least one enzyme with FBPase activity for gluconeogenic growth. Moreover, ΔglpXΔgpm2 lacked detectable FBPase activity at any substrate concentration that we tested (Fig. 6b), suggesting that the strain’s inability to grow on gluconeogenic substrates is due to disruption of gluconeogenesis at the FBPase reaction step. Consistent with this, FBPase activity was restored in ΔglpXΔgpm2 expressing either GLPX or GPM2 (Fig. 6b). The extent of complementation of FBPase activity with GLPX or GPM2 reflected the properties of each enzyme. Expression of GLPX in ΔglpXΔgpm2 provided lithium-sensitive FBPase activity that was only observed with 1.5 mM FBP, but not detected with 12 mM FBP, consistent with substrate inhibition of
Figure 5 | GPM2 has lithium-resistant FBPase activity. (a) FBPase activity progress curve for recombinant His-tagged GPM2. Data are mean ± s.d. of three technical replicates. Plotted line represents the non-linear fit for the three parameter Hill equation (r² = 0.99073255, s.e. of estimate = 0.0009). (b) Recombinant GPM2 FBPase activity in the presence of 12 mM FBP and varying amounts of lithium chloride. Data are mean ± s.d. of three technical replicates.

GLPX at high substrate concentrations. In contrast, expression of GPM2 in ΔglpXΔgpm2 generated lithium-resistant FBPase activity, and, consistent with the higher Kₘ of GPM2, this activity increased with higher FBP concentrations.

Gluconeogenesis is disrupted in ΔglpXΔgpm2. We next examined the metabolic state of ΔglpXΔgpm2 Mtb in the presence of a gluconeogenic carbon source using a filter-based metabolomics platform. Bacteria were first grown on 0.2% glucose for 5 days to obtain sufficient biomass and then transferred to a universally ¹³C-labelled (U-¹³C) carbon source (0.2% acetate or 0.2% glucose) for 24 h. When ΔglpXΔgpm2 was switched from glucose to U-¹³C-labelled acetate (Fig. 7), phosphoenolpyruvate (PEP) and the triose-phosphates (triose-P)—metabolites upstream of the FBPase-catalysed reaction in gluconeogenesis—accumulated. While FBP levels recovered metabolites upstream of the FBPase-catalysed reaction in pyruvate (PEP) and the triose-phosphates (triose-P)—accumulated. While FBP levels recovered.

Table 1 | Comparison of GPM2 and GLPX FBPase activity properties.

|                  | GPM2  | GLPX  |
|------------------|-------|-------|
| Enzymatic kinetics model | Allosteric sigmoidal | Michaelis–Menten |
| Kₘ (μM)          | 5.51±0.05 | 44±1    |
| kₐₘ (s⁻¹)        | 1.87±0.20 | 1.0±0.1 |
| h (s⁻¹)          | 3.02±0.10 | 0±0.05  |
| kₐₘ/Kₘ (s⁻¹·μM⁻¹) | 3.39×10⁴ | 2.27×10⁴ |

FBPase activity is required for Mtb virulence. We next evaluated whether FBPase activity and gluconeogenesis are required during an Mtb infection. In a mouse model of Mtb infection, ΔglpX was able to replicate and persist in lungs similar to the WT strain (Fig. 8a). In contrast, ΔglpXΔgpm2 failed to replicate in mouse lungs during the first 10 days of infection and began to die thereafter. ΔglpXΔgpm2 was effectively cleared from the host by 56 days post infection when the strain’s c.f.u. burden was below the limit of detection (4 c.f.u. per lung). While WT Mtb and ΔglpX were detected at comparable levels in mouse spleens at both 28 and 56 days post infection, we were unable to detect ΔglpXΔgpm2 at either time point (Fig. 8b). The clearance of ΔglpXΔgpm2 from mouse lungs was also evident from lung histopathology. Only mice infected with ΔglpXΔgpm2 lacked visible lung lesions at 56 days post infection (Supplementary Fig. 9). The severe attenuation of ΔglpXΔgpm2 in vivo indicates that both FBPase activity and gluconeogenesis are required for Mtb virulence. The attenuation of ΔglpXΔgpm2 in mouse lungs and size of upstream and downstream metabolites and ¹³C labelling of hexose-P and S7P. Thus, expression of either of the two enzymes with FBPase activity is sufficient for functional gluconeogenesis in Mtb. Notably, the restoration of gluconeogenic carbon flux through the FBPase reaction step upon expression of GPM2 supports this enzyme’s activity as an FBPase in vivo.

When ΔglpXΔgpm2 was grown on U-¹³C glucose (Supplementary Fig. 7), there were no significant changes in pool sizes or ¹³C labelling of metabolites in central carbon metabolism, indicating that glycolysis was not disrupted in ΔglpXΔgpm2. This is consistent with the ability of ΔglpXΔgpm2 to grow on glucose. Collectively, our metabolomic analyses suggest that loss of FBPase activity in ΔglpXΔgpm2 results in a specific disruption of gluconeogenesis at the FBPase-catalysed reaction, rendering Mtb unable to grow on gluconeogenic substrates.

Deletion mutants of some enzymes in the gluconeogenesis pathway, such as FBA and TPI, were not only unable to grow using fatty acids but died in the presence of this carbon source. When ΔglpXΔgpm2 was grown on U-¹³C glucose for at least 28 days (Supplementary Fig. 6b). Fatty acids were not toxic to ΔglpXΔgpm2 when provided in combination with glucose (Supplementary Fig. 8). On the contrary, ΔglpXΔgpm2 grew more robustly with 0.2% glucose and 0.1% acetate than with 0.4% glucose alone.
spleens could be fully complemented by expressing either GLPX or GPM2 in the ΔglpXΔgpm2 background. Thus, Mtb requires expression of at least one enzyme with FBPase activity to establish infection.

**Discussion**

Our work demonstrates that, in addition to GLPX, Mtb expresses a second FBPase, GPM2. The existence of a second FBPase in Mtb has been suggested before by transposon mutagenesis studies\(^24,25\). The inositol monophosphate phosphatase (IMPase) CYSQ (Rv2131c) was put forth as a possible second FBPase because the recombinant enzyme has FBPase activity \(\textit{in vitro}\)\(^31\). However, CYSQ has since been shown to function in sulfur metabolism as a 3'-phosphoadenosine-5'-phosphatase (PAPase)\(^32\). Not only does CYSQ have a higher \(k_{\text{cat}}/K_m\) with PAP as substrate than with IMP or FBP, but expression of CYSQ complemented the sulfite auxotrophy of \(E. \text{coli} \Delta \text{cysQ}\), demonstrating that this enzyme functions as a PAPase \(\textit{in vivo}\). Along with the other IMPase homologues—SUHB, IMPA and IMPC—CYSQ contains a sequence motif, which defines a phosphomonoesterase superfamily that includes lithium-sensitive IMPases and FBPases\(^21\).

While we initially considered the IMPase homologues as candidates for the second FBPase, our finding that ΔglpX FBPase activity was lithium resistant ruled out these candidates. There are species with both a Type I and a Type II FBPase (\(E. \text{coli}\))\(^12\) or a Type II and Type III FBPase (\(Bacillus subtilis\))\(^33\), but mycobacteria lack Type I and Type III FBPase homologues. Thus, the second FBPase activity likely derived from a non-classical FBPase. Proof-of-concept for an unrelated phosphatase supporting FBPase activity in the absence of a classical FBPase was previously demonstrated in \(E. \text{coli}\), where expression of an

![Figure 6](image-url)
alkaline phosphatase complemented the growth defect of Δfhp on glycerol. Our observations of gluconeogenic growth and FBPase activity in an FBPase mutant are similar to those reported for the yeast Yarrowia lipolytica. Deletion of the Type I FBPase in Y. lipolytica resulted in a partial growth defect on gluconeogenic carbon sources. Preliminary enzymology demonstrated that this mutant had detectable, lithium-resistant FBPase activity due to an ‘alternative phosphatase’ with a high $K_m$ for FBP. The second FBPase in this species remains to be identified. Nonetheless, it is apparent that functional redundancy between a classical FBPase and an alternative phosphatase can occur in other organisms. Our observation of similar results in Msm is consistent with the existence of a GPM2 homologue in this species: MSMEG_1926 (69% identity with GPM2). Thus, FBPase redundancy may extend to other mycobacterial species with GPM2 homologues.

The predicted function of GPM2 has changed over time. Initially, the protein was annotated based on homology to the E. coli 4'-phosphopantetheinyl transferase ENTD, which is involved in siderophore biosynthesis. An alternative classification placed the enzyme as a member of the cofactor-dependent phosphoglycerate mutase (dPGM) family from which the current GPM2 name is derived. Phosphoglycerate mutases operate in the gluconeogenesis pathway, driving the conversion of 2-phosphoglycerate to 3-phosphoglycerate via a 2,3-bisphosphoglycerate intermediate. Functional assays and structural analysis by Watkins and Baker showed that GPM2 more closely resembles members of the broad-specificity phosphatase subfamily within the dPGM family. The authors determined that GPM2 had negligible mutase activity and that its function was more accurately described as a broad-spectrum acid phosphatase. Because of GPM2’s ability to dephosphorylate a variety of substrates, it has been suggested that GPM2 serves as a phosphate scavenging enzyme, enabling Mtb to obtain phosphate from host molecules or its own metabolites inside the nutrient-limiting environment of the macrophage. Indeed, gpm2 expression was upregulated during phosphate starvation, suggesting that GPM2 might play a role during phosphate starvation. Our work describes a previously unknown function of GPM2. While GPM2 was once hypothesized to play a role in gluconeogenesis as a phosphoglycerate mutase, we now demonstrate that this enzyme maintains this pathway as an FBPase when GLPX is absent. This new function is consistent with its previously reported ability to act on phosphorylated sugars. Our findings neither support nor rule out GPM2’s role in maintaining the mycobacterial phosphate pool, but show that this
enzyme can serve a redundant role to a more specialized enzyme. Based on our work and that of Watkins et al., we propose that the function of GPM2 as a sugar phosphatase be recognized in the future with a new name for this enzyme: SUP1. Since gluconeogenesis is critical to Mtb's ability to establish infection, the conservation of GPM2 is likely a byproduct of the high selective pressure on Mtb to maintain a functional gluconeogenic pathway. Given our results, GPM2 could potentially compensate for the loss of other specialized phosphatases in other contexts, providing redundancy to other Mtb metabolic pathways that are critical to virulence.

Our enzymology reveals that GPM2 has different properties from those of the classical FBPase GLPX. With its lower $K_m$, GLPX has a higher affinity for FBP with optimal FBPase activity in the mid-micromolar substrate concentration range. The higher $K_m$ of GPM2, on the other hand, confers optimal FBPase activity in the millimolar range. Although GPM2 has a lower affinity for FBP, the enzyme's higher $k_{cat}$ makes it a robust FBPase when supplied with enough substrate. The different kinetics of GLPX and GPM2 suggest that these enzymes likely play different roles as FBPases. GPM2's allosteric sigmoidal kinetics suggests that this enzyme's robust FBPase activity at high FBP concentrations is substrate-tunable. In contrast, GLPX's Michaelis–Menten kinetics make this enzyme less sensitive to substrate concentration, resulting in a core low level activity at lower FBP concentrations. While GPM2 can provide sufficient FBPase activity in the absence of GLPX, it is not entirely clear how this occurs. Since the two enzymes have different substrate affinities, GLPX and GPM2 are not active as FBPases at the same substrate concentration. Under WT Mtb conditions, FBP levels are likely optimized for GLPX over GPM2 FBPase activity. Loss of GLPX in Mtb, however, leads to a buildup of FBP that allows GPM2 FBPase activity, and effectively maintains gluconeogenesis in the absence of the classical FBPase. Such substrate-level control of enzymatic reactions has been reported before. In E. coli, a transaldolase deletion mutant can unexpectedly grow on xylose due to an accumulation of S7P that supports alternative 7-phosphosedohepturo-kinase activity from the 6-phosphofructokinase PFKA, driving a functional bypass of the blocked transaldolase reaction step. The FBP concentration in Mtb is undetermined. In E. coli, levels of FBP can range from 15 mM when cultured on glucose to < 150 μM when cultured on acetate. The FBP concentration in B. subtilis was 4.3 mM when grown on glycerol as the sole carbon source. Millimolar FBP concentrations similar to those observed in E. coli and B. subtilis would be sufficient to support GPM2 FBPase activity.

We sought to address whether gluconeogenesis is required for Mtb virulence. By deleting both glpX and gpm2 in Mtb, we generated a strain that lacks detectable FBPase activity and, as a consequence, suffers from defective gluconeogenesis as evidenced from in vitro growth assays and metabolomics analysis. The attenuation of ΔglpXΔgpm2 in the mouse model indicates that Mtb requires gluconeogenesis for virulence. The bacterial burden of ΔglpXΔgpm2 was significantly lower than that of WT as early as 10 days post infection, suggesting that Mtb requires gluconeogenesis early during the course of an infection. Glycolysis, which is intact in ΔglpXΔgpm2, was not sufficient to support replication of ΔglpXΔgpm2 in vivo. These results are consistent with an earlier study that showed that glycolysis was dispensable for establishing infection but played a role in persistence. We also observed killing and eventual clearance of ΔglpXΔgpm2 in mouse lungs, indicating that gluconeogenesis is necessary for survival of Mtb in the host. The mechanism resulting in death of ΔglpXΔgpm2 in vivo awaits future study. Disruption of gluconeogenesis at the FBPase reaction step in ΔglpXΔgpm2 might kill the bacterium through the accumulation of a toxic upstream metabolite. For instance, accumulation of FBP could be a source of toxicity, as was suggested for an E. coli FBA deletion mutant. However, while ΔglpXΔgpm2 accumulates FBP and other metabolites upstream of the FBPase reaction when grown on acetate in vitro, this strain does not die in this condition. On the contrary, growth of ΔglpXΔgpm2 on glucose was enhanced by acetate, suggesting that Mtb may still be able to co-catabolize these substrates in the absence of FBPase activity. Therefore, in vivo killing of ΔglpXΔgpm2 is likely not due to accumulation of phosphorylated metabolites. The discrepancy between the in vitro and in vivo survival of ΔglpXΔgpm2 mirrors the results obtained with ΔpckA, another mutant with a gluconeogenesis-specific defect. Loss of gluconeogenesis may contribute to the in vivo attenuation of both strains by producing a common set of metabolic changes that predispose the bacteria to killing via anti-microbial mechanisms in the host.

GLPX has been considered as a drug target for TB chemotherapy. Humans lack a Type II FBPase, allowing design of a species-specific drug that would selectively target Mtb gluconeogenesis. While our study highlights Mtb gluconeogenesis as critical to the pathogen's ability to survive in the host, it also suggests that blocking this pathway at the FBPase step would be challenging. A drug targeting GLPX would not be sufficient to inhibit growth or kill Mtb, as the pathogen can utilize GPM2 to maintain gluconeogenic carbon flux. Furthermore, GLPX and GPM2 belong to different enzyme families. FBPase activity of the phosphomonoesterase family member GLPX involves metal cofactors coordinating and activating a water nucleophile to attack the phosphorus 1 atom. In contrast, the phosphatase activity of the DPGM family member GPM2 does not involve a metal cofactor and uses a phosphohistidine catalytic intermediate instead. These differences in the catalytic mechanisms of GLPX and GPM2 FBPases will hamper design of a single inhibitor against both enzymes.

In conclusion, we report previously undescribed redundancy in FBPase activity in Mtb that complicates the design of
FBPase-targeting drugs. We also demonstrate the importance of gluconegenesis for \textit{Mtb} virulence. A better understanding of how defective gluconegenesis contributes to clearance of \textit{Mtb} in vivo could identify alternative targets for new TB drugs.

**Methods**

**Strains and culture conditions.** \textit{Mtb} H37Rv (obtained from the Trudeau Institute) was cultured in Middlebrook 7H9 containing 0.05% Tween-80, 0.5% bovine serum albumin fraction V, 0.2% glucose, 0.085% NaCl and incubated standing at 37 °C with 5% CO\textsubscript{2}. For AglpXgpm2, Middlebrook 7H9 containing 0.05% Tween-80, 0.5% bovine serum albumin fraction V, 0.4% glucose and 0.085% NaCl was used. For solid agar, Middlebrook 7H10 agar with 0.5% glycerol and 10% OADC enrichment (0.5% bovine serum albumin fraction V, 0.2% glucose, 0.085% NaCl, 0.006% oleic acid and 0.003% catalase at final concentration) was used. For AglpXgpm2, Middlebrook 7H10 agar with 0.4% glucose, 0.5% bovine serum albumin fraction V and 0.085% NaCl was used. \textit{Mtb} growth curves were performed using carbon-defined Sauton’s minimal media containing 0.05% potassium phosphate monobasic, 0.05% magnesium sulfate heptahydrate, 0.2% citric acid, 0.005% ferric ammonium citrate, 0.05% ammonium sulfate, 0.001% insulin, 0.05% tryptophol adjusted to pH 7.4. For enzyme activity assays, strains were cultured in Middlebrook 7H9 containing 0.05% Tween-80, 0.5% bovine serum albumin fraction V, 0.085% NaCl and either 0.2% glycerol or 0.4% glucose as the sole carbon source. For metabolomics analysis of AglpXgpm2, \textit{Mtb} was seeded onto 0.22 μm pore size filters at OD\textsubscript{580} = 0.1 and incubated on Middlebrook 7H10 agar with 0.2% glycerol, 0.5% bovine serum albumin fraction V, and 0.085% NaCl and incubated at 37 °C with 5% CO\textsubscript{2} for 5 days. Filters were then transferred to plates of similar composition with different carbon sources (0.2% glucose, 0.2% U\textsubscript{13C} glucose, 0.2% acetate or 0.2% U\textsubscript{13C} acetate) and incubated at 37 °C for 5 days. Filters were then transferred to plates with either 0.2% glycerol or 0.2% U\textsubscript{13C} glycerol and incubated for 16h before harvesting.

\textit{M. mcat} (ATCC 700084) was cultured in Middlebrook 7H9 containing 0.05% Tween-80, 0.2% glycerol, 0.4% glucose, and incubated at 37 °C with 5% CO\textsubscript{2} and light shaking. At 24h before harvesting, the growth was monitored by quenching in acetone/methanol/d\textsubscript{H2O} (40:40:20) on dry ice followed by mechanical lysis by bead beating with 0.1 mm zirconia/silica beads and clarification using a 0.2 μm filter as described previously\textsuperscript{49}. For metabolomics analysis of AglpXgpm2, filters were placed on Middlebrook 7H10 agar with 0.2% glycerol, 0.5% bovine serum albumin fraction V, and 0.085% NaCl and incubated at 37 °C with 5% CO\textsubscript{2} for 5 days. Filters were then transferred to plates with either 0.2% glycerol or 0.2% U\textsubscript{13C} glycerol and incubated for 16h before harvesting.

Carbon sources were provided at the described concentration as w/v or v/v. 10 μg ml\textsuperscript{-1} kanamycin and 25 μg ml\textsuperscript{-1} zeocin were used for selection where appropriate.

**Mutant generation and validation.** All vectors used for mutant generation and complementation were constructed using Gateway Cloning Technology (Invitrogen). Primer sequences are provided in Supplementary Table 3. Mutant genotypes were confirmed by PCR and Southern blot analysis.

AglpX was generated using allelic exchange and specialized transducing phage pKX500, which was constructed using the same vector used to complement \textit{Mtb} D\textsubscript{glpX} with a C-terminal Flag tag. A 800 bp fragments corresponding to regions upstream and downstream of mmseg_5239 (glpX homologue) were amplified by PCR and cloned into pSC284-loxp flanking the hygromycin resistance gene. We generated pSC284-loxp, a derivative of pSC284 (gift from Jeff S. Cox), that contains loxp sites flanking the hygromycin cassette. The plasmid was digested with PacI and packaged into the 

**FBPase activity assay.** Protein lysates were prepared from 50 ml cultures in specific media at OD\textsubscript{580} = 1. Briefly, cells were washed overnight in 20 ml Tris-HCl pH 7.7, and resuspended in 1 ml of the same buffer containing 0.1 μg ml\textsuperscript{-1} lysosome protease inhibitor, EDTA-free cocktail. Lysis was achieved by bead beating with 0.1 mm zirconia/silica beads three times at 4,500 r.p.m. for 30 s with 30 s samples kept on ice for 5 min between beadings. Beads and cell debris were removed by centrifugation (11,000g, 10 min, 4 °C) and the supernatant was passed through a 0.22 μm filter.

24 h activity measurements were performed using a previously described spectrophotometric assay that couples G6P production to conversion of NADPH\textsuperscript{+} to NADP\textsuperscript{+}, which can be detected as a change in absorbance at 340 nm (refs 23,51). Reactions were performed in cuvettes with a 1 ml final reaction volume containing 20 mM Tris-HCl pH 7.7, 8 mM MgCl\textsubscript{2}, 50 mM KCl, 1 mM NADPH\textsuperscript{+}, 1 μM yeast G6P dehydrogenase (Sigma-Aldrich), 2.5 μM yeast phosphoglucoisomerase (Sigma-Aldrich) and either 50 μg ml\textsuperscript{-1} total protein lysate or 0.05 μg ml\textsuperscript{-1} purified recombinant GPM2. Reactions were incubated at 30 °C for 5 min before starting the reactions by adding FBP. Reactions were followed using a Uvikon XL UV/VIS spectrophotometer to measure absorbance at 340 nm over time. Data were analyzed by linear regression using LabPower Jr software, version 2.06-0106S. For enzymology of GPM2, FBPs were concentrated from 50 ml 7H9 media containing kanamycin and glucose 20-fold in 50 ml 7H9 media containing kanamycin and glucose.

**Immunoblot analysis.** Protein lysates were prepared in the same manner as described for the FBPase activity assay. 50-100 μg total protein were separated by SDS-PAGE and then transferred to nitrocellulose membranes for probed with pNit-RecET-sacB grown to OD\textsubscript{580} = 1 and incubation was carried out for 8 h at 37 °C. After incubation, the culture was used to prepare competent \textit{E. coli} cells, which were then transformed with 500 ng of the gpm2 KO Cassette PCR product and plated on 7H10 agar containing zeocin and glucose as the sole carbon source. The genotype-validated AglpXgpm2 clone was cured of the recombinering plasmid by plating on 7H10 agar containing 10% sucrose, zeocin and glucose as the sole carbon source and then by testing for kanamycinity sensitivity. The complementation of AglpXgpm2 with \textit{pNit-RecET-sacB} was achieved using pKT-P121-glpx, which was also used to complement AglpX. Complementation of AglpXgpm2 with gpm2 was achieved using pGM-EK-psy60-gpm2-FLAG (SD), the same vector used to complement AglpX\textsuperscript{Δmut}.

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FBPase activity purification by HPLC. AglpX protein lysate was prepared in the same manner as used for the FBPase activity assay. Four steps of liquid chromatography were performed using a AKTAPAFFF liquid chromatography system (GE Healthcare). After each step, active fractions were identified using the described FBPase activity assay and then pooled for further purification. All HPLC columns were obtained from GE Healthcare. At a minimum, the Buffer A used in each purification step consisted of 25 mM HEPES (pH 7.4) and 0.5 mM β-mercaptoethanol. First, anion exchange chromatography was performed with a 5 ml HiTrap Q Sepharose FF column using a gradient from 0 to 6 M NaCl over 25 column volumes. Next, the pooled active fractions were diluted 1:1, with 2 M ammonium sulfate and hydrophobic interaction chromatography was performed with a 1 ml HiTrap Phenyl Sepharose FF (high sub) column using a gradient from 1 to 0 M ammonium sulfate over 20 column volumes. Active fractions were pooled and concentrated using a Microcon YM-3 (3000 MWCO) centrifugal filter device (Millipore) before performing size-exclusion chromatography with a Superose 6 10/300 GL column using Buffer A with 650 mM NaCl as the mobile phase. Next, cation exchange chromatography was performed with a Mono Q 5/50 GL column using a
gradient from 0.1 to 0.4 M NaCl over 25 column volumes. The final active fractions were run on a 15% SDS–PAGE gel and stained using the Invitrogen Silver Quest gradient from 0.1 to 0.4 M NaCl over 25 column volumes. The final active fractions were cut, processed and analysed by liquid chromatography–reversed phase nano-LC-MS/MS (NCS3500RS Nano and Q-Exactive, Thermo Scientific). Tandem MS data was extracted using ProteomeDiscoverer v1.3 (Thermo, Bremen, Germany) and queried against UniProt Mtb H37Rv database using Mascot 2.3 (Matrixscience, London, UK). Peptides with a Percolator-based false discovery rate of 1% or better were reported.

Expression and purification of recombinant GPM2. pET300-NT-gpm2, a vector for inducible expression of an N-terminus 6-His-tagged GPM2 in E. coli, was constructed using Gateway Cloning Technology (Thermo, Bremen, Germany) and queried against UniProts based false discovery rate of 1% or better were reported.

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Author contributions
U.G., J.M. and S.E. designed the research; U.G., J.M. and S.C. performed the research; U.G., J.M., S.C., H.E., K.R., L.P.S.dC. and S.E. analysed the data; and U.G. and S.E. wrote the paper.

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