The *Mycobacterium tuberculosis* Rv1099c gene encodes a GlpX-like class II fructose 1,6-bisphosphatase

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There are now abundant data indicating that *Mycobacterium tuberculosis* uses fatty acids as a carbon source *in vivo*. A key enzyme in gluconeogenesis, missing in the original annotation of the *M. tuberculosis* genome, is fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11). The authors have shown that *M. tuberculosis* Rv1099c, a *glpX* homologue, can complement *Escherichia coli* mutants lacking FBPase. The protein encoded by Rv1099c was shown to have FBPase activity. Rv1099c was expressed at significant levels in *M. tuberculosis*, and may encode the major FBPase of this pathogen.

INTRODUCTION

Improving our understanding of the intracellular environment in which the pathogen *Mycobacterium tuberculosis* lives is one approach to identifying targets for the development of new rational control measures. A number of observations point to the importance of fatty acids as carbon and energy sources in members of the *M. tuberculosis* complex (Fig. 1). Thus a mutant lacking isocitrate lyase, part of the glyoxylate shunt, has a reduced persistence phenotype in a mouse model (McKinney *et al.*, 2000). In the related *Mycobacterium bovis*, the pyruvate kinase gene is non-functional (Garnier *et al.*, 2003), so the glycolysis pathway is inactive. In *M. bovis* BCG, a mutant lacking *pckA*, the gene for phosphoenolpyruvate carboxykinase (one of the two non-reversible steps in gluconeogenesis), is attenuated *in vivo* (Liu *et al.*, 2003), and the *pckA* gene is up-regulated during growth in macrophages (Dubnau *et al.*, 2002). Another study showed that *M. tuberculosis* cells present in sputum contain lipophilic inclusions (Garton *et al.*, 2002), while *Mycobacterium microti* uses palmitic acid preferentially over simple carbon sources (Wheeler & Ratledge, 1988). Finally, the TraSH methodology that identifies mutations responsible for poor growth in bacteria following transposon mutagenesis (Sasseti & Rubin, 2003) identified a number of fatty acid degradation

genes, the glyoxylate shunt genes, and *pckA* as being essential *in vivo*. Taken together, these findings emphasize the importance of the glyoxylate shunt and gluconeogenesis in using the carbon from fatty acids for biosynthesis of the sugars that are abundant in the cell wall and glycolipids of mycobacteria.

The second irreversible step in gluconeogenesis is fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11). Although the completion of the *M. tuberculosis* genome sequence allowed the identification of genes that were predicted to encode enzymes for most central metabolic pathways (Cole *et al.*, 1998), no FBPase was assigned. Four classes of FBPases have been identified based on primary structure (Rittmann *et al.*, 2003). These are *Escherichia coli* Fbp-like (class I) (Sedivy *et al.*, 1984), *E. coli* GlpX-like (class II) (Donahue *et al.*, 2000), *Bacillus subtilis*-like (class III) (Fujita *et al.*, 1998) and dual function FBPase-inositol monophosphate phosphatases (class IV) (Verhees *et al.*, 2002). A fifth class has been identified in the Archaea, which may represent the 'true' FBPase in these organisms (Rashid *et al.*, 2002). In *E. coli*, the major FBPase is a class I member encoded by the *fbp* gene, for which there is no detectable homologue in the actinomycetes. It has recently been reported that a minor class II FBPase is encoded in *E. coli* by the *glpX* gene, which is part of the glycerol 3-phosphate regulon (Donahue *et al.*, 2000). The protein encoded by the *M. tuberculosis* Rv1099c gene shows 43 % identity to *E. coli* GlpX. In this work, the Rv1099c gene was cloned. Results of genetic and biochemical analyses revealed that the Rv1099c gene is likely to encode the missing mycobacterial FBPase.

Abbreviations: COGs, Clusters of Orthologous Groups (database); FBPase, fructose 1,6-bisphosphatase; GST, glutathione S-transferase; RTq-PCR, real-time quantitative PCR.

Fig. 1. Lipid utilization by *M. tuberculosis* leading to lipoglycan synthesis via gluconeogenesis. The genes *glpX* and *pckA* which code for enzymes catalysing irreversible steps are shown, as well as *fum*, which lies next to *glpX* in the *M. tuberculosis* genome. The unidirectional arrows are drawn to emphasize the directions of reactions in gluconeogenesis, and do not indicate whether reactions are inherently uni- or bidirectional. The grey arrows indicate steps in the TCA cycle that would be used with glucose, but not with fatty acids, as a carbon source.

METHODS

Bacterial strains and plasmids. *E. coli* JLD2404 (Δ *fbp287*), JLD2402 (*glpX*::*Spc*^R Δ *fbp287*), and JB108 (BL21[λ DE3 (*lacUV5-T7 gene1*)] Δ *fbp287*) have been described before (Donahue *et al.*, 2000). *M. tuberculosis* H37Rv was used for RNA analysis. Plasmid vectors used were pET15b (p_{T7}-His-tag; Novagen) and pGEX-KG (p_{tac}-GST; AmershamPharmacia). In pGEX-KG, the glutathione *S*-transferase (GST) fusion protein is induced directly by IPTG, while in pET15-b, the fusion protein is expressed by an IPTG-inducible T7 RNA polymerase encoded by a lysogenic phage. pFM142 and pFM149 carry *M. tuberculosis* *Rv1099c* cloned into pET15-b and pGEX-KG, respectively, and are described in this study.

***E. coli* culture conditions.** Strains were grown in Luria broth (LB) supplemented with antibiotics as needed or in M9 minimal media (Sambrook *et al.*, 1989) containing 0.2% glucose or 0.4% glycerol. *E. coli* DH5 α (Life Technologies) was used for all plasmid constructions.

Cloning of *Rv1099c*. *M. tuberculosis* *Rv1099c* was cloned into expression vectors using the Tuberculist-predicted start codon (position 35 in Fig. 2). The *Rv1099c* coding sequence was amplified by PCR using primers *Rv1099c*-NdeI (GGAATTCCATATGGAGCTGGTCCGGGT) and *Rv1099c*-XhoI (TGAATTCGAGGGCAATGGGTACACG). These

primers introduced an *Nde*I site at the 5' end and an *Xho*I at the 3' end to allow the gene to be cloned in-frame into the expression vector pET-15b. Primers *Rv1099c*-PvuII (GGATCAGCTGATGGAGCTGGTCCGGGT) and *Rv1099c*-EcoRI (CGGAATTCGGGCAATGGGTACACG) were used to introduce a *Pvu*II site at the 5' end and an *Eco*RI at the 3' end to allow the gene to be cloned in-frame into the expression vector pGEX-KG. The primers were each used at 300 nM final concentration. PCR was carried out using the Expand High Fidelity PCR system (Boehringer Mannheim) with *M. tuberculosis* DNA as the template and DMSO at 2%. The temperature cycle used was: an initial 3 min at 94 °C to denature high-G+C DNA; 10 cycles of 45 s at 94 °C, 1 min at 63 °C and 1 min at 72 °C; 25 cycles of 45 s at 94 °C, 1 min at 63 °C and 1 min at 72 °C (this last increasing by 20 s per cycle); and finally an extension step of 7 min at 72 °C to complete primer extension. The PCR products were cleaved with the appropriate restriction enzymes and cloned into the vectors pET-15b or pGEX-KG. In this way, plasmids expressing the *M. tuberculosis* *Rv1099c* gene were generated, as both His-tag and GST-fusion constructs.

Enzyme preparation and assay. Strains of *E. coli* were grown with aeration at 37 °C in LB medium to the exponential phase, induced with IPTG (0.1 mM), and incubated with shaking at room temperature for 3 h. Bacteria were harvested by centrifugation, washed and the pellets were resuspended in 2–4 ml 25 mM Tris/HCl (pH 7.3) containing 2 mM dithiothreitol. This suspension was sonicated for 40 s at 50% amplitude with a 5 mm diameter probe (Sonics & Materials VibraCell), and the sonicate clarified by centrifugation (12 000 g, 5 min). Virtually all enzyme activity was in the supernatant fractions. GST activity was assayed using the AmershamPharmacia GST detection module according to the manufacturer's instructions. FBPase activity was assayed at 37 °C by measuring the fructose 6-phosphate produced in an assay coupled to phosphoglucose isomerase and glucose-6-phosphate dehydrogenase by following the production of NADPH (Rashid *et al.*, 2002). Preliminary attempts to purify fusion proteins using HiTrap Chelating HP or GS Trap FF columns (AmershamPharmacia) according to the manufacturer's instructions resulted in loss of FBPase activity. Therefore, clarified cell extracts were used for the enzyme work described below. All enzyme extracts and preparations were desalted using AmershamPharmacia PM10 columns equilibrated and eluted with 25 mM Tris/HCl (pH 7.3) containing 2 mM dithioerythritol according to the manufacturer's instructions.

To estimate the *K_m*, the concentration of fructose 1,6-bisphosphate was varied from 2 mM (the concentration routinely used elsewhere; see Rashid *et al.*, 2002) down to 3 μ M. Fructose 1,6-bisphosphate was used at 20 μ M in all experiments designed to test for potential inhibitors, including LiCl, that were included at the concentrations mentioned in Results. The potential inhibitors were mixed with extracts and incubated for 5 min before adding substrate to start the reaction.

Quantitative PCR. RNA was prepared from an exponential (7-day) rolling culture of *M. tuberculosis* H37Rv (Betts *et al.*, 2002), and cDNA synthesis was carried out using Superscript II (Invitrogen) according to the manufacturer's protocol. Real-time quantitative PCR (RTq-PCR) reactions were set up using the DyNAmo SYBR Green qPCR kit (MJ Research) and RTq-PCR was performed using the DNA Engine Opticon 2 System (Genetic Research Instrumentation). Reactions containing 1 $\times$ DNA Master SYBR Green I mix, 1 μ l cDNA product and 0.4 μ M of each primer in 20 μ l were set up on ice. Samples were heated to 95 °C for 10 min before cycling for 35 cycles of 95 °C for 30 s, 60 °C (*Rv1099c*) or 62 °C (*sigA*) for 20 s, and 72 °C for 20 s. Fluorescence was captured at the end of each cycle after heating to 80 °C to ensure the denaturation of primer-dimers. The experiment was repeated three times using cDNA from each of two independent RNA preparations.

Fig. 2. A multiple sequence alignment of actinomycete class II FBPAases and *E. coli* GlpX. The putative class II FBPAases for *M. tuberculosis* (Mtb), *M. smegmatis* (Msm), *M. avium* (Mav), *M. leprae* (Mlp), *Streptomyces coelicolor* (Sco), *Corynebacterium diphtheriae* (Cdi), and *E. coli* (Eco) GlpX and *C. glutamicum* (Cgl) Fbp were aligned with CLUSTALW (Thompson *et al.*, 1994) using default settings. The CLUSTALW alignment was shaded using the CHROMA program (Goodstadt & Ponting, 2001). Fully conserved residues are in white on a black background; other residues are shaded according to their physicochemical properties. Invariant positions are indicated by asterisks (*) below the alignment, while highly conserved and weakly conserved positions are indicated by colons and periods, respectively. The Eco sequence was obtained from SWISS-PROT (accession no. P28860), the Cgl and Sco sequences were obtained from GenBank (accession nos 19552240 and 21223420 respectively), the Mtb sequence was obtained from the Tuberculist server (<http://genolist.pasteur.fr/Tuberculist/>; gene name *Rv1099c*), the Mlp sequence was obtained from the Leproma server (<http://genolist.pasteur.fr/Leproma/>; gene name ML1946), the Msm and Mav sequences were derived from unpublished genomic sequences obtained from The Institute for Genomic Research website (<http://www.tigr.org/>), and the Cdi sequence was obtained from the Sanger Centre (Cerdono-Tarraga *et al.*, 2003) (also available from GenBank: accession no. 38199795). The exact start residue of the proteins is known only for *Syneococcus* PCC7942 (Tamoi *et al.*, 1996) (which corresponds to the predicted *E. coli* start), and for *C. glutamicum* (Rittmann *et al.*, 2003). For all other proteins, we have predicted the start codon, which may not necessarily be the same as that given when using the aforementioned accession numbers, on the basis of mutual homologies. The (probably incorrect) start position for the Mtb and Mlp sequences (as described in the Tuberculist and Leproma databases) is indicated by an 'M'. The location of IMP1 Prosite motif (<http://www.expasy.ch/prosite> accession no. PS00629), is indicated by a bar above the alignment. The motif is nearly present in the mycobacterial putative class II FBPAases, as can be seen from the list of valid motif residues shown under the alignment (x = any residue valid): only one mismatch is found (position 121, underlined). However, there are two mismatches to the Sco sequence, and three mismatches to the Eco GlpX, the Cgl Fbp and the Cdi putative FBPAase II.

RESULTS

Comparison of *glpX* orthologues and their genomic contexts

The *Rv1099c* gene is homologous to *E. coli glpX*. An alignment of predicted GlpX proteins from several actinomycetes,

as well as *E. coli*, is shown in Fig. 2. We suggest that *Rv1099c* is a class II FBPAase; it has 43 % identity (62 % similarity) to *E. coli* GlpX, and low similarity to members of the other FBPAase classes. In the Clusters of Orthologous Groups (COGs) database (Tatusov *et al.*, 2001), *Rv1099c* is clustered with other class II (GlpX-like) FBPAases.

Rv1099c is the first gene in a cluster of three genes that we predict is an operon (see Fig. 3). The second gene, *fum* (30 bp downstream), encodes the only predicted fumarase in the genome, which is presumed to function in the citric acid cycle (Fig. 1). Its location next to *Rv1099c* is conserved in all the sequenced mycobacterial genomes, and suggests a functional relationship between these two genes. *Rv1097c*, the start of which overlaps the end of *fum* in *M. tuberculosis*, is a putative Gly/Pro-rich membrane protein with unknown

Fig. 3. Comparative genome analysis of actinomycete *glpX* genes. The genetic structure of regions containing the *glpX* gene are shown for *M. tuberculosis* (*Mtub*), *M. smegmatis* (*Msm*), *M. avium* (*Mav*), *M. leprae* (*Mlep*), *Corynebacterium glutamicum* (*Cglu*), *C. diphtheriae* (*Cdip*) and *S. coelicolor* (*Sco*). Black arrows, *glpX* gene; white arrows, genes showing homology to those in the *M. tuberculosis* *phoH2-Rv1101c* region; grey arrows, genes not showing homology to those in the *M. tuberculosis* *phoH2-Rv1101c* region; white hatched arrow (*ML1948*), as for grey arrows but pseudogene. Gene names/numbers arrows refer to the gene identification for that species except for *M. avium*, which has not yet been annotated, and *M. tuberculosis* designations are used. Numbering of genes refers to the relevant species: thus 1100c is short for *Rv1100c*, 1945 is short for *ML1945*.

function that is not conserved in other species. A conserved hypothetical gene, *Rv1100*, is transcribed divergently from this putative operon, and its conserved synteny in other genomes is suggestive of a related function.

The intergenic region between the divergently transcribed *Rv1099c* and *Rv1100* genes is currently annotated at 100 bp. We suggest, however, that the start of the coding sequence (MELV...) predicted for *Mtb Rv1099c* in GenBank (accession no. 15608239) and in Tuberculist Release 5 (<http://genolist.pasteur.fr/TubercuList/>) is misannotated (Fig. 2), because the homology with *E. coli* GlpX extends several amino acids upstream to a different methionine, and homology to the *Corynebacterium glutamicum* Fbp (confusingly the homologue of *E. coli* GlpX, and not of *E. coli* Fbp, i.e. a class II FBPase) extends further still. Critically, experimental analysis of the *C. glutamicum* protein confirms that this conserved region is translated (Rittmann *et al.*, 2003), contrary to the assignment in the published sequence. This would mean that the *M. tuberculosis* protein is likely to start 66 (VSAH...) or 102 (MTAE...) base pairs upstream. Alignment with the *Mycobacterium avium* sequence, which has a potential start analogous to the -102 but not the -66 start, suggests that the -102 start is correct. Similarly, we predict the *Mycobacterium leprae* start codon to be 57 bases upstream of that predicted by the Leproma database Release 2 (<http://genolist.pasteur.fr/Leproma/>). *Mycobacterium smegmatis* has an analogous methionine to the start codon of the *C. glutamicum*.

This reassignment of the start of *M. tuberculosis Rv1099c* affects the predicted start for the divergently transcribed *Rv1100*, as it overlaps the newly assigned start codon for *Rv1099c*. While it is possible that both promoters lie within coding sequences, it is more likely that there is an intergenic gap. The corynebacterial *Rv1100* homologues are located further upstream of *glpX* (~200 bp), and have only one potential start site upstream of conserved regions (*C. glutamicum*: VAEK...). *M. tuberculosis Rv1100* has a potential GTG start codon in a similar position in the aligned proteins; this aligns with the likely start in *M. smegmatis* (MTTQ...), and we propose that this is the genuine start codon. This would leave the intergenic gap between *Rv1099c* and *Rv1100* at 57 bp. We suggest new translational starts for the *M. leprae* homologues using similar logic: *glpX* (*ML1946*) will start 81 bp upstream of the current assignment at base 2332629 (old start, MELV...; new start, MTAE...), and the *Rv1100* orthologue *ML1945* will start 84 bp of the current assignment at base 2332549 downstream (old start, MVND...; new start, VTFE...).

Complementation of *E. coli* mutants

It is possible to test for FBPase activity by genetic complementation, as an *E. coli fbp* mutant is unable to grow on medium with glycerol as sole carbon source (Donahue *et al.*, 2000). We therefore cloned *Rv1099c* into two expression vectors, so that it would be expressed either with a short N-terminal His-tag (pFM142), or fused to the C-terminal

Fig. 4. Growth pattern of the glucose-requiring *E. coli* strains. JLD2402 (1), and JLD2402 transformed with the pGEX-KG vector (2) or pFM149 (3) were streaked onto minimal agar containing glucose or glycerol as carbon source.

end of GST (pFM149). These constructs were introduced into *E. coli* strains JLD2402, which lacks both *fbp* and *glpX*, and JLD2404, which lacks only *fbp*. Antibiotic-resistant transformants were selected and then plated onto minimal agar plates containing glucose or glycerol as carbon source, and IPTG to induce expression of Rv1099c. All of these strains grew on glucose agar, whereas the control strains only grew on glycerol agar (results with JLD2402 and pFM149 are shown in Fig. 4). Complementation was not expected from the pET15b-based clone pFM142, because of the lack of a host T7 polymerase gene, but was detected; our experience is that this can occur due to read-through from other promoters. These results confirm that Rv1099c has FBPAse activity. For historical reasons, the clones were made

using the start site predicted by Tuberculist. The fact we obtained activity indicates that if the protein does have an extended N-terminus as we predict, this region is not critical for function.

FBPAse activity in complemented *E. coli* mutants

pFM142 and pFM149 were transformed into *E. coli* JB108, a BL21(λ DE3)-based strain that lacks *fbp*, and allows both pGEX and pET15b-based constructs to be expressed. A spectrophotometric coupled-enzyme assay was used to measure the FBPAse activity in cell-free extracts (Table 1). The *E. coli* JB108 (Δ *fbp*) host gave low background levels, and introduction of pFM142 or pFM149 resulted in elevated levels of FBPAse. Note that although strain JB108 has a functional chromosomal *glpX* gene, we showed that FBPAse levels in strain JLD2402 (Δ *fbp* Δ *glpX*) were not significantly different from those in strain JLD2404 (Δ *fbp*) (Table 1), supporting earlier observations that although *E. coli* GlpX has FBPAse activity, the gene is expressed at low levels (Donahue *et al.*, 2000).

We determined that the K_m of the *M. tuberculosis* recombinant enzyme for fructose 1,6-bisphosphate was 15 μ M (three determinations, range 12–17 μ M), using *E. coli* cell-free extracts. The K_m values obtained were similar for the two types of fusion protein made in this study, suggesting that the type of fusion construct used did not affect the affinity of the FBPAse for its substrate. The effects of phosphoenolpyruvate, AMP, ADP, citrate and Li^+ on the FBPAse activity of both the GST fusion and the His-tagged proteins were tested. None of the metabolites had

Table 1. FBPAse activity in *E. coli* strains expressing mycobacterial GlpX proteins

Abbreviations: sp. act, specific activity; *n*, number of determinations; IU, international enzyme units (1 unit converts 1 μ mol substrate min^{-1} ; therefore 1 mIU converts 1 nmol min^{-1}); NA, not applicable.

Strain/construct*	FBPAse sp. act. [nmol min^{-1} (mg protein) $^{-1}$] $\dagger$	<i>n</i>	Sp. act. (mIU FBPAse/IU GST)
BL21(λ DE3)	18.3 $\pm$ 5.75	5	NA
JB108 [Δ <i>fbp</i>]	0.29 $\pm$ 0.18	4	NA
JB108(pGEX-KG)	0.01 $\pm$ 0.03	3	0‡
JB108(pET15b)	0 $\pm$ 0.19	2	NA
JB108(pFM149)	15.3 $\pm$ 1.79	6	11.4
JB108(pFM142)	18.3 $\pm$ 0.35	2	NA
JLD2404 [Δ <i>fbp</i>]	−0.04 $\pm$ 0.05§	3	0.21
JLD2402 [Δ <i>fbp</i> Δ <i>glpX</i>]	0.23 $\pm$ 0.11	3	0.47
JLD2404(pFM149)	17.7 $\pm$ 0.42	3	11.1
JLD2402(pFM149)	26.0 $\pm$ 7.0	4	13.0

*Square brackets indicate relevant genotype of strain.

$\dagger$ Means $\pm$ SEM.

‡Zero here denotes that the FBPAse activity was not significantly above zero.

§Negative values are possible as Δ [NADPH] without fructose 1,6-bisphosphate is subtracted from Δ [NADPH] with fructose 1,6-bisphosphate in a simultaneous assay.

any effect up to 1 mM, but 1.1 mM Li^+ gave 50 % inhibition and 10 mM Li^+ gave over 90 % inhibition (not shown).

Gene expression levels in *M. tuberculosis*

We carried out RTq-PCR experiments to determine the level of expression of *Rv1099c* mRNA in exponential cultures of *M. tuberculosis* in Middlebrook 7H9 medium containing OADC supplement and Tween 80. Expression levels were normalized to those of *sigA* mRNA, and calculated based on the RNA used for reverse transcription. We showed that in mid-exponential-phase growth in supplemented Middlebrook 7H9 medium (which contains both glucose and Tween 80 as carbon sources), the level of *Rv1099c* mRNA is 0.49 (95 % confidence interval 0.39–0.63) that of *sigA*.

DISCUSSION

Although no FBPase was annotated in the genomes of any of the actinomycetes when this work started, the ability of these organisms to grow on gluconeogenic substrates such as fatty acids or glycerol requires FBPase to be present in order to provide the hexose needed for synthesis of their abundant cell envelope glycans and mannolipids. In other organisms, particularly the Archaea, where no FBPase was annotated during whole-genome analyses, other members of the carbohydrate phosphatase superfamily (<http://scop.mrc-lmb.cam.ac.uk/scop/>; release 1.65) have been shown to encode active FBPases. In *M. tuberculosis*, this superfamily comprises the *impA*, *suhB*, *impC* (*Rv3137c*), *cysQ* and *Rv1099c* genes.

We show here that *M. tuberculosis* *Rv1099c* has FBPase activity with saturable kinetics, and a K_m at 15 μM that is similar to the affinity of bacterial class II FBPases for fructose biphosphate. For example, the *C. glutamicum* enzyme has a K_m of 14 μM and the *E. coli* GlpX has a K_m of 35 μM . Lithium inhibits by interfering with the Mg^{2+} binding site of sugar phosphatases (Chen & Roberts, 1998), so its effect on the *M. tuberculosis* FBPase activity is consistent with this. The lack of effect of any of the intermediates is surprising as it is expected that GlpX needs to be regulated, for instance to avoid futile cycling when phosphofructokinase is active. However, their effects need to be investigated further on purified, possibly cleaved fusion protein before drawing any firm conclusions about regulation.

We suggest that *Rv1099c* may be the major FBPase of *M. tuberculosis*; this is supported by the recent report where the GlpX homologue of *C. glutamicum* was characterized (Rittmann *et al.*, 2003). The authors showed that it contained FBPase activity, and demonstrated that a mutant lacked any detectable FBPase activity. The corynebacterial *glpX* mutant was incapable of growth on gluconeogenic substrates, which substantiates the observation that no other FBPase is predicted by the genome sequence of this

organism. The *C. glutamicum* gene was accordingly named *fbp*; we, however, propose that *Rv1099c* be called *glpX* in order to avoid the implication that it is orthologous to the *E. coli* *fbp* gene; the *glpX* name is also used in the COGs database.

Interestingly, all the mycobacterial class II FBPases shown in Fig. 2 have a near-perfect Prosite (<http://www.expasy.ch/prosite>) inositol monophosphate phosphatase IMP1 motif (PS00629), although they have no IMP2 motif (PS00630). This may suggest a closer homology of the mycobacterial class II FBPases to IMPases than other members of the family. No activity with inositol 1-phosphate could be attributed to *Rv1099c* under the conditions examined (data not shown).

We demonstrated that *Rv1099c* is expressed in the cell. mRNA levels approximately half of the level of *sigA*, the major sigma factor of the cell, were detected. The fact that it is expressed is also indicated by the identification of the protein in 2D-PAGE analysis (<http://www.mpiib-berlin.mpg.de/2D-PAGE/EBP-PAGE/index.html>).

Support for a key biological role for the *M. tuberculosis* *glpX* gene comes from independent concurrent research using a genome-wide transposon-based method (TraSH) for identifying genes that are required for growth in different conditions (Sassetti *et al.*, 2001; Sassetti & Rubin, 2003). The data suggest that the *fum* gene (Figs 1 and 3) is an essential gene for axenic growth in the presence of both glycolytic (glucose) and gluconeogenic (oleic acid) carbon sources together (Middlebrook 7H9 + OADC + Tween 80). Mutants deficient in *Rv1100* (which is conserved syntenically with *Rv1099c* in mycobacteria and corynebacteria) grew slowly, but it appeared that mutants deficient in *Rv1099c* grew normally. However, a comparison between axenic and *in vivo* growth showed that mutants in *Rv1099c* were among the most severely attenuated in a mouse model. This was confirmed in separate experiments showing that an *Rv1099c* transposon mutant is highly attenuated *in vivo* (Sassetti & Rubin, 2003). The extreme attenuation of the *glpX* mutant is consistent with the essential role of gluconeogenesis for conversion of lipid carbon into cell wall glycan (Fig. 1). These data, together with the demonstration that a *C. glutamicum* *fbp* mutant has lost all detectable FBPase activity, suggest that *glpX* encodes the major FBPase in *M. tuberculosis*.

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