Glycolytic and Non-glycolytic Functions of Mycobacterium tuberculosis Fructose-1,6-bisphosphate Aldolase, an Essential Enzyme Produced by Replicating and Non-replicating Bacilli

Background: New drugs active against persistent Mycobacterium tuberculosis are needed.

Results: The fructose-1,6-bisphosphate aldolase (FBA-tb) is essential for growth of M. tuberculosis, is expressed by replicating and non-replicating bacilli, and displays plasminogen binding activity.

Conclusion: FBA-tb is an essential TB enzyme that might also play a role in host/pathogen interactions.

Significance: FBA-tb shows potential as a novel anti-TB therapeutic target.
Class II Aldolase of M. tuberculosis

the tubercle bacillus. The resulting latent stage of infection is associated with a few bacteria surviving for years in a latent or "semidormant" state with low metabolic activity (3–9). Persistent bacteria appear to be resistant to common chemotherapy and may be reactivated, resulting in active disease. Although the physiological state of persistent M. tuberculosis bacilli during latent human infection is largely unknown, there is now substantial evidence that M. tuberculosis undergoes important metabolic changes to ensure a constant supply of carbon and energy from alternative sources and pathways (1). Analysis of patient autopsy specimens, transcriptional profiling of bacteria recovered from tuberculous lesions, and studies with various inhibitors and knock-out mutants of M. tuberculosis all indicate that persistent bacilli encounter low oxygen tension, which may contribute in part to their non-replicating state. They are also subject to nutrient deprivation, which obliges them to divert carbon from host-derived fatty acids into gluconeogenesis (4–14). Targeting pathways involved in the adaptation of M. tuberculosis to latent infection may thus represent a promising approach to the eradication of persistent bacilli.

The search for drug therapies against persistent TB has led to our interest in class II fructose-1,6-bisphosphate aldolase (FBA-tb), a key enzyme of glycolysis/gluconeogenesis more abundantly produced and/or secreted by M. tuberculosis grown under low oxygen tension (15–17). FBA-tb is a homotetrameric enzyme dependent on zinc for activity (18), and the crystal structures of three different protein substrate/product complexes were recently solved (19). Fructose-bisphosphate aldolases (FBAs) (EC 4.1.2.13) are enzymes involved in glycolysis where they reversibly catalyze cleavage of fructose 1,6-bisphosphate (FBP) to yield dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. They are also active in gluconeogenesis and the Calvin cycle where they catalyze the reverse condensation. FBAs are divided into two classes depending on their reaction mechanism. Class I aldolases are present in higher organisms (animals and plants), green algae, and a few prokaryotes. They form a Schiff base intermediate between the carbonyl substrate (FBP or dihydroxyacetone phosphate) and a lysine residue of the active site. In contrast, class II aldolases require a divalent metal ion (usually zinc or cobalt) to polarize the carbonyl group of the substrate (FBP or dihydroxyacetone phosphate) and to stabilize the carbonan intermediate during catalysis. They are mainly found in lower organisms such as bacteria (eubacteria and archaebacteria), fungi, protozoa, and some green algae. Class I and class II enzymes show almost no sequence similarities and are assumed to have arisen from separate evolutionary origins (20). The absence of class II FBAs from mammalian cells and the specificity of their structure and catalytic mechanism thus offer the opportunity to design drugs that selectively inhibit microbial class II enzymes without affecting the gluconeogenetic and glycolytic pathways of the host. This study was undertaken with the goals of assessing the therapeutic potential of FBA-tb and providing biochemical and structural bases for future drug design.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Escherichia coli DH5α, the strain used for cloning experiments, was propagated in LB broth (pH 7.5) (BD Biosciences). M. tuberculosis H37Ra (ATCC 25177) was grown in Middlebrook 7H9 broth (Difco) supplemented with ADC and 0.05% Tween 80 or on solid Middlebrook 7H11 agar supplemented with OADC. Kanamycin, streptomycin, and hygromycin were added to final concentrations of 20, 20, and 50 μg mL⁻¹, respectively. When required, 2% sucrose and anhydrotetracycline were added to the media.

For growth on defined carbon sources, 7H9-tyloxapol (0.025%) broth and 7H11 agar containing 0.5% BSA and 0.085% NaCl were used and supplemented with ADC, 40 mM succinate, or 40 mM acetate. Low oxygen tension experiments were performed according to Wayne and Hayes (21) with appropriate controls to check for oxygen depletion except that Sauton medium (without detergent) instead of Dubos medium was used to allow FBA-tb activity to be measured in the culture filtrate (i.e. in a medium devoid of albumin).

Allelic Replacement at fba-tb Locus of M. tuberculosis—The Ts-sacB method (22) was used to achieve allelic replacement at the fba-tb (MRA_0372) locus of M. tuberculosis H37Ra (100% identical in sequence to Rv0363c of M. tuberculosis H37Rv). The M. tuberculosis gene and flanking regions were PCR-amplified from M. tuberculosis H37Rv genomic DNA, and a disrupted allele, fba-tb:kan, was obtained by inserting the kanamycin resistance cassette from pUC4K (Amersham Biosciences) into the SaII restriction site of the suicide plasmid pPR27-fba-tbKK, the construct used for allelic replacement. pNIP40b-fba-tb, one of the plasmids used for complementation, was obtained by first cloning the PCR-amplified coding sequence of the fba-tb gene into the pVV16 expression plasmid (23) and then transferring the fba-tb gene under control of the phsp60 promoter into the XbaI restriction site of the integrative plasmid pNIP40b (24). pGMCS-10M-P1-fba, the integrative rescue plasmid used in gene silencing experiments, was constructed by cloning the PCR-amplified fba-tb gene into pDO23A (25) by BP recombination (Invitrogen), resulting in pEN23A-fba. After confirmation that fba-tb was cloned without mutations, an LR recombination (Invitrogen) was performed with pEN41A-T10M, pEN12A-P1, and pDE43-MCS (25) to generate pGMCS-10M-P1-fba. pEN41A-T10M and pEN12A-P1 are Gateway entry plasmids containing tetR10 (26) and P<sub>gyrE</sub>tetO (27), respectively. pDE43-MCS is a Gateway destination plasmid that includes the integrase and attP site of the mycobacteriophage L5 and a streptomycin resistance gene. BP and LR Clonase™ mixtures were purchased from Invitrogen and used according to the manufacturer’s instructions. Allelic replacement at the fba-tb locus was confirmed by PCR using primers located outside the rescue copy of the gene carried by pNIP40b-fba-tb and pGMCS-10M-P1-fba and outside the disrupted copy of fba-tb used in the allelic exchange experiment. Primer sequences are available upon request.

Fba-tb Expression and Purification—fba-tb was PCR-amplified from M. tuberculosis H37Rv genomic DNA using primers fba_Ndel_fwd (5’-ggaattctatatgcctatcgcaacgcccgagg-3’) and
fba_BamHI_rev (5’-ccggatctcaggtgtagcttcg-3’), and the PCR product was ligated into the Ndel and BamHI restriction sites of the expression vector pET29a (Novagen). FBA-tb production in E. coli BL21(DE3) (Novagen) was induced by adding 1 mM isopropyl-1-thio-D-galactopyranoside (MP Biomedicals) and allowing the cells to grow for 16 h at 18 °C. Cells were harvested and resuspended in 50 mM Tris-HCl, pH 8.0 (solution A) containing protease inhibitors (Complete EDTA-free, Roche Applied Science) and disrupted by sonication, and nucleic acids were degraded by the addition of Benzonase (Novagen). The soluble fraction was applied to a Q Sepharose column (GE Healthcare) equilibrated with solution A, and the column was washed with solution A until no absorbance at 280 nm was detected. Elution was performed with a linear gradient of 0–0.5 M NaCl in solution A at 5 ml min⁻¹. Fractions containing FBA-tb were pooled, and a solution of saturated ammonium sulfate was added dropwise until a final concentration of 30% was reached. The solution was incubated at 4 °C for 1 h, and the soluble fraction was loaded onto a phenyl-Sepharose column (GE Healthcare) equilibrated with solution A with 1.5 M ammonium sulfate. The column was washed with solution A containing 1.5 M ammonium sulfate until no absorbance at 280 nm was detected. Elution was performed by a linear gradient of 1.5–0 M ammonium sulfate in solution A. Fractions containing FBA-tb were pooled and concentrated over a Vivaspin 20 membrane (10,000-Da cutoff; Sartorius). The concentrate was then dialyzed against solution A and loaded at 1 ml min⁻¹ onto a Superdex 200 column (GE Healthcare) equilibrated with solution A containing 150 mM NaCl. Fractions were collected, pooled, concentrated to 50 mg ml⁻¹, and stored at −80 °C.

**Polycyclon Antibody Production, Immunoblotting, and Flow Cytometry**—Polycyclon antibodies against purified FBA-tb were produced in an outbred New Zealand White rabbit (Western Oregon Rabbit Co., Philomath, OR). Subcellular fractions from M. tuberculosis H37Rv grown in glycerol-alanine-salt medium and Mycobacterium leprae purified from armadillo tissues were obtained from the TB Vaccine Testing and Research Materials Contract (NIAID, National Institutes of Health N01-AI-40091) and the Leprosy Research Support Contract (NIAID, National Institutes of Health N01-AI-25469), respectively. The polyclon anti-FBA-tb antibody and the secondary goat anti-rabbit antibody conjugated to alkaline phosphatase were used at a concentration of 1:3,500 and 1:5,000, respectively, in immunoblotting experiments carried out with the M. tuberculosis and M. leprae extracts. The polyclonal rabbit anti-PimA antibody was used at a concentration of 1:3,000.

For the cell surface localization of FBA-tb by flow cytometry, polyclonal rabbit IgG was purified using the Dynabeads® Protein A and DynaMag™-2 kits (Invitrogen) according to the manufacturer’s recommendations. M. tuberculosis H37Ra bacilli grown in Sauton medium were dispersed by gentle vortexing with 5-mm glass beads for 2 min. Bacteria were resuspended in 1 ml of PBS buffer containing 0.1% BSA, 2% fetal bovine serum (FBS), and 5 µg of anti-FBA-tb IgG; incubated overnight at 4 °C; washed three times with PBS; and incubated for another 2 h in the dark with goat anti-rabbit IgG-Alexa Fluor 647 conjugate (Invitrogen) diluted 1:2,000 in PBS, 0.1% BSA, and 2% FCS. Bacteria were then washed twice in PBS and finally resuspended in 0.4 ml of PBS containing 4% formalde-hyde. Untreated bacteria and bacteria incubated with non-immunized (control) rabbit serum, the primary anti-FBA-tb antibody alone, or the secondary antibody alone were used as controls. Samples were analyzed for fluorescence using a CyAn flow cytometer. Under the experimental conditions used, only the FBA-tb protein accessible to the antibodies at the surface of the bacilli but not the intracellular protein should be stained. Cells were detected using forward and log side scatter dot plots, and a gating region was set to exclude cell debris and bacterial aggregates; 20,000 bacterial cells were analyzed.

**Auramine-Rhodamine Staining**—Lung sections from IFN-γ-KO mice and guinea pigs infected with M. tuberculosis Erdman and M. tuberculosis H37Rv for 18 and 28 days, respectively, were stained with TB fluorescent stain kit T (BD Biosciences) according to the manufacturer’s instructions except that counterstaining was performed with hematoxylin QS (Vector Laboratories, Inc., Burlingame, CA) for ~5 s. The slides were washed with double distilled H2O and mounted using ProLong® Gold antifade reagent (Invitrogen) (28).

**Immunohistochemistry**—Formalin-fixed, paraffin-embedded lung tissue sections were digested with 30 units ml⁻¹ a-chromopeptidase and 1 mg ml⁻¹ lysozyme in 10 mM Tris, pH 8.0 for 40 min at 37 °C. Endogenous peroxidases were blocked using a 3% H2O2 solution for 40 min at 25 °C. Slides were then placed in a Retriever™ 2100 pressure cooker (Pickell Laboratories, Amsterdam, The Netherlands) at 121 °C for 15 min and then incubated with 1% goat serum for 30 min. The slides were incubated with a 1:500 dilution of the serum containing the rabbit anti-FBA-tb antibody and a mouse anti-GroES antibody (Antibody SA-12, Colorado State University TB Vaccine Testing and Research Materials Contract) along with 1% goat serum for 16 h at 4 °C. The slides were then incubated with a 1:100 dilution of horseradish peroxidase-labeled goat anti-rabbit antibody and also an Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) in 1% goat serum for 40 min at 25 °C. The signal was amplified using a 1:400 dilution of tyramide-Alexa Fluor 568 in amplification buffer (Invitrogen). Nuclei were stained with DAPI (Invitrogen) at 200 ng ml⁻¹ for 10 min and mounted with ProLong Gold antifade mounting medium (Invitrogen).

**Human Plasminogen Binding and Activation Assays**—The binding of plasminogen (Plg) to FBA-tb, BSA, and fibrinogen (Sigma) was analyzed by ELISA in Immulon 2HB plates (Thermo Scientific). Wells coated with 2 µg of FBA-tb or control proteins were incubated with different concentrations of Plg in PBS-Tween 20 with 1% BSA for 1 h at 37 °C. Mouse anti-Plg (Pierce) was used as the first antibody, and goat anti-mouse IgG HRP-conjugated antibodies (Thermo Scientific) were used as the secondary antibodies. Peroxidase activity was revealed with 3,3′,5,5′-tetramethylbenzidine substrate solution (Thermo Scientific), and absorbance at 450 nm was measured. A chromogenic assay using Chromozym (Roche Applied Science) was used to monitor the activation of Plg (1 µg) into plasmin at A405 nm in the presence of FBA-tb (2 µg), 0.0875 units (24 ng) of streptokinase (Sigma), and other control proteins (2 µg) with or without added human tissue Plg activator (tPA) (0.025 µg) (Calbiochem). Assays were performed either
in solution or in the presence of a fibrin matrix (prepared in the
wells from fibrinogen with human thrombin (Sigma)), and
assay conditions were as described earlier (29). In studies aimed
at analyzing the regulation of plasmin activity by α2-antiplasmin
(Innovative Research), FBA-tb (2 μg) and human Plg (1 μg) were
first preincubated for 1 h at 37 °C to allow FBA-tb-Plg complexes to
form prior to the addition of tPA (0.025 μg) and finally α2-antiplas-
min (2 μg) at which point protease activity was monitored over
time. All assays were run in duplicates or triplicates, and the results
of representative experiments are shown.

**FBA-tb Enzymatic Assays**—The coupled assay used to mea-
sure the enzymatic activity of FBA-tb is based on that of Rich-
ards and Rutter (30). The standard reaction mixture contained
50 mM HEPES, pH 7.5, 80 μM NADH (Fluka), 5 units ml−1
triose-phosphate isomerase (Sigma), 2 units ml−1 α-glycero-
phosphate dehydrogenase (Sigma), 20 μM FBP (Fluka), and 0.2–
1.5 μg ml−1 purified enzyme in a total volume of 1 ml. 15 μg of
*M. tuberculosis* H37Ra cellular extracts or capsular or culture
filtrate proteins replaced the purified protein in the assays
directed on whole mycobacterial cultures. Capsular and
culture filtrate proteins were prepared as described (31).
Cellular extracts were prepared by breaking *M. tuberculosis* H37Ra cells using a FastPrep® instrument (MP Biomedicals)
and centrifuging the lysates at 4,000 rpm for 20 min at 4 °C
to remove the unbroken cells. A correction was made for the
NADH oxidase and putative class I aldolase activities of the
crude extracts by running assays alongside in which FBP was
omitted or EDTA (1 mM) was added. All experiments were
repeated at least twice, and activity was measured in duplica-
tate on each sample.

**Crystallization and Data Collection**—FBA-tb crystals were
grown by vapor diffusion from a 1:1 mixture of protein solution
(10 mg ml−1 initial protein concentration made up in 25 mM
Tris HCl, pH 7.0) and precipitant buffer (20% PEG 8000, 1.8 M
Li2SO4, and 50 mM Tris/HOAc, pH 7.5), and 4–μl drops were
equilibrated at 23 °C against 1-ml reservoirs of precipitant solu-
tion. Crystals grew in 2 weeks. Enzymatic assays revealed com-
plete inhibition of FBA-tb under conditions of the crystalliza-
tion buffer (1.8 M Li2SO4 and 50 mM Tris/HOAc, pH 7.5). Resid-
ual activity at saturating substrate concentration was
<1% of maximal activity. Inhibition was corroborated by mon-
itoring hydrazone formation at 240 nm in a chemical assay
based on reaction of hydrazone with the nascent aldehyde (32).

FBA-tb crystals were soaked in TD3 inhibitor buffer (mother
liquor plus 10 μM TD3 compound) for 10 min. The structure of
TD3 is identical to that of compound 1 shown in Ref. 33. Prior to
data collection, crystals were cryoprotected by transfer through a
cryobuffer solution (compound TD3 plus 10% glycerol) and
immediately flash frozen in a stream of gaseous N2 cooled to 100 K.
Diffraction data were collected from single crystals at beamline
X29 of the National Synchrotron Light Source (Brookhaven
National Laboratory, Upton, NY) with an Area Detector Systems
Corp. Quantum 315r detector to 1.9-Å resolution. All data sets
were processed with XDS (34) and SCALA from the CCP4 suite
(35), and the results are summarized in Table 2. A control data set
without TD3 was also collected to 2.35-Å resolution under identi-
cal experimental conditions.

**Structure Solution and Refinement**—FBA-tb structures with
and without TD3 were solved by molecular replacement with
the program AutoMR (Phenix suite) (36) using native FBA-tb in
complex with FBP (19) (Protein Data Bank code 3elf) as the
search model. The structure in complex with compound TD3
belongs to space group C2 (dimer of homodimers in the asym-
metric unit). The best solution was used as the starting point for
refinement of the liganded structure and was solved by iterative
rounds of refinement (simulated annealing and minimization)
with Phenix and model building using Coot (37). The MolPro-
bity server (38) and the Coot validating tools were used to opti-
mize the structures during the refinement. Water molecules
were automatically added by Phenix in the initial rounds and
manually near the end of the refinement. Loop regions (resid-
ues 165–180) in each subunit were associated with regions of
weak electron density. In the final round of refinement, the
occupancy of TD3 was adjusted to take into account competi-
tion by sulfate ions for the TD3 phosphate oxyanion binding
sites. Refinement of the unbound native structure used sulfate
ions positioned coincident with phosphate oxyanion binding
sites of TD3. The loop region corresponding to residues 211–
221 and adjacent to residues 165–180 was associated with weak
electron density in all subunits of the native FBA-tb and was not
modeled into the structure.

Ligand modeling was based on interpretation of electron
density shapes of 2Fo – Fc and Fo – Fc annealed omit maps and
using the Phenix.elbow command for topology and parameter
generation. Binding by compound TD3 was readily discernable
and associated with clearly defined electron densities in the
active site in two subunits of the same homodimer. In the
remaining homodimer, active site electron densities showed no
evidence for detectable binding by TD3. Occupancies of the
catalytic zinc ion closely matched that of TD3 and were set
equal to TD3 occupancy in the final round of refinement. Dif-
ference electron density (Fc – Fc) annealed omit maps calcu-
lated in the final round of refinement confirmed identical bind-
ing of TD3 in protomers. Final model statistics calculated with
Phenix, MolProbity, and SFCHECK (39) are shown in Table 2.
The coordinates and structure factors of native FBA-tb (Protein
Data Bank code 4a21) and in complex with TD3 (Protein Data
Bank code 4a22) have been deposited with the Protein Data
Bank. The final structure of the native FBA-tb and FBA-tb in
complex with compound TD3 was refined to Rcryst (Rfree) values
of 0.211 (0.261) and 0.191 (0.229). The corresponding Luzzati
atomic coordinate errors were estimated at 0.32 and 0.23 Å,
respectively. Ramachandran analysis with the MolProbity
server placed at least 98.0% of non-glycine and non-proline res-
ides of the two structures in the most favorable region with the
remainder found in allowed regions, attesting to good model
geometry in the structures.

**Structure Comparisons**—Superimpositions were performed
with the super command in PyMOL with use of Ca atom coor-
dinates of identical regions of amino acid sequences or by LSQ
fit in Coot. Root mean square deviations are reported based on
superimposition of equivalent Ca atoms in FBA-tb structures
and for positional comparisons of compounds based on super-
imposition of equivalent non-hydrogen atoms.
RESULTS AND DISCUSSION

*M. tuberculosis* Aldolase Is an Essential Glycolytic and Gluconeogenetic Enzyme of *M. tuberculosis*—The requirement of FBA-tb for *M. tuberculosis* growth was examined genetically by knocking out the chromosomal copy of fba-tb in the presence or absence of a rescue copy of this gene carried by an integrative plasmid. Attempts to inactivate fba-tb in *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv in the absence of a rescue copy of this gene yielded no candidate mutant at the last selection step of the procedure, suggesting that, unlike the situation in *Neisseria meningitidis* (40) but similar to those in *E. coli*, Streptomyces, and *Candida albicans* (41–43), the class II FBA gene of *M. tuberculosis* is required for growth even under optimal laboratory growth conditions where both glucose and oleic acid are present in the culture medium. As proposed in the case of *E. coli* (44), it is likely that this requirement is related to the role of FBA-tb in preventing the toxic effect of FBP accumulation in the cells. FBP is indeed an allosteric effector of several enzymes in central carbon metabolism (pyruvate kinase, phosphoenolpyruvate carboxylase, ADP-glucose pyrophosphorylase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase) whose deregulation is likely to affect cell growth (44–46). Inhibition of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase by high levels of FBP in the absence of FBA-tb for instance would prevent flux through the oxidative branch of the pentose phosphate pathway (46) and inhibit reductive biosyntheses as well as induce oxidative stress.

Allelic exchange experiments were then conducted in a merodiploid strain, H37Ra/pNIP40b-fba-tb, carrying an integrated rescue copy of fba-tb. The choice of using *M. tuberculosis* H37Ra is justified by the fact that the H37Ra strain is considered a class II organism yet requires FBA-tb for growth like its virulent counterpart, *M. tuberculosis* H37Rv, with which it shares the exact same glycolytic/gluconeogenetic and pentose phosphate pathway enzymes (100% identical in their primary sequence) (47). Mutants in which the wild-type chromosomal copy of the gene was inactivated were easily isolated using this merodiploid strain, confirming the requirement of fba-tb for growth of *M. tuberculosis* on 7H11-OADC-sucrose plates (Fig. 1A).

To assess the requirement of FBA-tb under gluconeogenetic conditions, which might be experienced by tubercle bacilli during the persistence stage of the infection (14), a conditional *M. tuberculosis* H37Ra mutant was constructed in which the chromosomal copy of fba-tb was knocked out and replaced by a wild-type copy of this gene expressed in cis from a tetracycline-inducible promoter. The successful construction of the conditional mutant, hereafter referred to as H37RaΔfba/pGMCS-10M-P1-fba, was confirmed by PCR. Gene silencing experiments in 7H9 medium containing various carbon sources mapping upstream or downstream of FBP in the glycolytic pathway revealed that the growth of H37RaΔfba/pGMCS-10M-P1-fba in glucose- and succinate-containing media was strictly dependent upon the induction of fba-tb expression with anhydrotetracycline (Fig. 1B). FBA-tb is thus an essential enzyme under both glycolytic and gluconeogenetic conditions.

*M. tuberculosis*, like *E. coli*, has been reported to display both class I and class II FBA activities in vitro (15, 16). In the presence of a class I enzyme with a redundant role in glycolysis/gluconeogenesis, the reason for the essentiality of the class II aldolase of *M. tuberculosis* is thus unclear. The fact that we were not able to detect any significant class I aldolase activity under any of the growth conditions tested in this study (see further section) raised doubts as to its existence. Also, failure to categorically identify a class I aldolase gene in *M. tuberculosis* despite the availability of a growing number of genome sequences of this bacterium further supported its absence from *M. tuberculosis*. Indeed, BLAST searches for orthologs of class I FBA genes from prokaryotic (*E. coli* and *Thermococcus gammatolerans*) and eukaryotic (human FBA isozymes A, B, and C) sources in the genome of *M. tuberculosis* H37Rv/H37Ra all yielded negative results. A class I enzyme whose N-terminal sequence matches that of protein MSMEG_3507 from *Mycobacterium smegmatis* mc²155 was purified to near homogeneity from a *M. smegmatis* isolate (48) and found to share extensive sequence similarities with other prokaryotic class I enzymes. However, an analysis of the distribution of this gene within the *Mycobacterium* genus failed to identify any orthologs beyond fast growing non-tuberculous *Mycobacterium* species and the *Mycobacterium avium* complex.

FIGURE 1. fba-tb is required for *M. tuberculosis* growth. A, evidence for allelic replacement at the fba-tb locus of *M. tuberculosis* H37Ra in the presence of a rescue copy of the gene integrated in the chromosome. Allelic exchange mutants were rescued with fba-tb expressed from the hsp60 promoter in pNIP40b-fba-tb. Allelic replacement at the fba-tb chromosomal locus was confirmed by PCR (see “Experimental Procedures”). WT, wild-type *M. tuberculosis* H37Ra. The wild-type 2.4-kb amplification signal is replaced by a 3.6-kb fragment in the allelic exchange mutants (Δ) due to the insertion of a 1.2-kb kanamycin resistance cassette. MWM, molecular weight marker. B, growth of *M. tuberculosis* H37Ra wild-type (squares) and *M. tuberculosis* H37RaΔfba/pGMCS-10M-P1-fba (circles) in the presence of glucose (under the form of ADC supplement) or succinate as carbon sources. Bacteria were inoculated at an initial A₆0₀nm of 0.01 and cultured at 37 °C with constant stirring in 7H9-tyloxapol broth with 40 mM succinate or ADC supplement in the presence (filled symbols) or absence (open symbols) of 100 ng ml⁻¹ anhydrotetracycline. Abs, absorbance.
Production of Active Form of FBA-tb and Evidence for Its Surface Exposure in M. tuberculosis—With the aim of studying the expression and subcellular localization of FBA-tb in M. tuberculosis grown under different in vitro and in vivo conditions, the FBA-tb protein was produced and purified from E. coli, and polyclonal anti-FBA-tb antibodies were raised in rabbit.

FBA-tb is a homotetrameric 344-amino acid protein constituting a 144-kDa molecule (18). An untagged form of FBA-tb was produced because N-terminal tags have been found to affect dramatically the solubility and activity of FBA-tb produced in E. coli probably by disrupting its quaternary structure (18, 49). Moreover, we found a C-terminal hexahistidine-tagged form of FBA-tb to be inactive when expressed in M. smegmatis. The untagged protein was produced as a soluble form in E. coli BL21(DE3) and purified to near homogeneity (18, 49). Moreover, we found a C-terminal hexahistidine-tagged form of FBA-tb to be inactive when expressed in M. smegmatis.

TABLE 1
Total and subcellular distribution of class II fructose-1,6-bisphosphate aldolase activity of M. tuberculosis bacilli grown under various axenic conditions

| Growth condition | Total FBA-tb activity | Subcellular fraction | Distribution of the FBA-tb activity in the total culture | Specific FBA-tb activity in each subcellular fraction |
|------------------|-----------------------|----------------------|--------------------------------------------------------|--------------------------------------------------------|
| 7H11-OADC       | 13 pmol FBP/min/µg protein | Cells 100% |                                         | pmol FBP/min/µg protein |
| 7H11-ADC        | 17 pmol FBP/min/µg protein | Cells 100% |                                         | pmol FBP/min/µg protein |
| 7H11-acetate     | 15 pmol FBP/min/µg protein | Cells 100% |                                         | pmol FBP/min/µg protein |
| Sauton-O_2 depletion | 11.3 ± 1.8 pmol FBP/min/µg protein | Cells 95% | 11 ± 1.6 pmol FBP/min/µg protein | 16 ± 5.4 pmol FBP/min/µg protein |
| Sauton-oxygenated | 10.7 ± 0.7 pmol FBP/min/µg protein | Cells 96% | 11 ± 0.6 pmol FBP/min/µg protein | 4 ± 1.8 pmol FBP/min/µg protein |
| Sauton-tyloxapol |                        |                      |                                          |                                          |
| Early log phase (T1) | 12.9 ± 2.0 pmol FBP/min/µg protein | Cells 99% | 13 ± 2.1 pmol FBP/min/µg protein | 4 ± 0 pmol FBP/min/µg protein |
| Midlog phase (T2)   | 13.9 ± 3.9 pmol FBP/min/µg protein | Cells 99% | 14 ± 4.0 pmol FBP/min/µg protein | 4 ± 0.12 pmol FBP/min/µg protein |
| Late log phase (T3)  | 13.9 ± 0.3 pmol FBP/min/µg protein | Cells 98% | 14 ± 0.3 pmol FBP/min/µg protein | 5 ± 1.7 pmol FBP/min/µg protein |
| Stationary phase (T4) | 11.9 ± 1.4 pmol FBP/min/µg protein | Cells 96% | 12 ± 1.4 pmol FBP/min/µg protein | 8 ± 0.6 pmol FBP/min/µg protein |

FIGURE 2. Flow cytometry analysis of axenically grown M. tuberculosis bacilli for FBA-tb surface exposure. M. tuberculosis H37Ra bacilli grown as a surface pellicle in Sauton medium were gently dispersed with glass beads and submitted to flow cytometry analysis for FBA-tb surface localization as described under “Experimental Procedures.” Bacteria were either untreated, treated with control rabbit serum followed by anti-rabbit IgG-Alexa Fluor 647, or treated with anti-FBA-tb antibodies followed by anti-rabbit IgG-Alexa Fluor 647. The histogram area in “R” represents the population of fluorescently labeled bacilli. SS, side scatter; FS, forward scatter.

6 P. M. Gest and M. Jackson, unpublished results.
tuberculosis H37Rv (supplemental Fig. S2). FBA-tb thus appears to translocate across the different compartments of the bacterial cell. The presence of antibody-accessible FBA-tb at the cell surface of the bacterium was further confirmed by direct enzyme activity measurements and by probing intact M. tuberculosis H37Ra bacilli with anti-FBA-tb antibodies followed by flow cytometry analysis (Table 1 and Fig. 2). Untreated bacteria and bacteria treated with control rabbit serum followed by anti-rabbit IgG-Alexa Fluor 647 produced weak fluorescence signals (1.4 and 18.5 mean fluorescence intensities, respectively), whereas cells treated with anti-FBA-tb followed by anti-rabbit IgG-Alexa Fluor 647 conjugate demonstrated a clear shift in fluorescence signal (104 mean fluorescence intensity), confirming the cell surface localization of FBA-tb (Fig. 2).

95% of the M. tuberculosis H37Ra bacilli grown as an undisturbed surface pellicle in Sauton medium were found in the R region, suggesting that the majority of the bacterial population had FBA-tb present on the cell surface. The finding of FBA-tb at the cell surface and in culture filtrates, although surprising in view of the apparent lack of secretion signals in the protein and its glycolytic/gluconeogenetic function, is consistent with the reported surface exposure of class II FBP aldolases from several other bacterial and fungal pathogens (40, 50–54) and earlier proteomics observations on M. tuberculosis (17, 55, 56).

FBA-tb Is Expressed by Replicating and Non-replicating Bacilli during Adaptation to Stationary Phase, Low Oxygen Tension, and Changes in Carbon Sources—Because of its involvement in gluconeogenesis and reported induction and/or oversecretion under low oxygen tension (15–17), we next sought to assess the potential regulation of fba-tb in M. tuberculosis grown under various axenic conditions including some thought to mimic the physical environment encountered by the bacilli during persistence in vivo. Enzyme assays were performed on cellular extracts, surface extracts, and culture filtrates of M. tuberculosis grown in the presence of glucose or fatty acids as carbon sources, under high or low oxygen tension, and at various stages of growth (Table 1). Culture filtrates and capsular fractions were checked for cell lysis by immunoblot with antibodies directed against PimA (a cytosolic GDP-Man-utilizing mannosyltransferase) (23) (supplemental Fig. S3).

Results confirmed the production of an active FBA-tb enzyme under all conditions tested including low oxygen tension (21). Interestingly, no significant up- or down-regulation of the overall class II aldolase activity of the cultures was found whatever the growth conditions tested (Table 1). Although surprising in light of what had been reported earlier for other glyoxylate cycle and gluconeogenetic genes (6, 12, 13, 57), our results are consistent with those of some 30 transcriptomics studies performed on M. tuberculosis bacilli grown under various stress conditions (e.g. inside macrophages and in the presence of SDS, drugs, NO, low oxygen tension, low iron, low nutrient, and various mutant backgrounds). Overall, the percentage of class II aldolase activity found in the culture filtrates never exceeded 4% of the total FBA-tb activity of the culture, and that found in the capsular surface-exposed material of surface pellicle-grown bacteria represented less than 7% of the total activity.
FBA-tb Is Expressed during Host Infection—To assess fba-tb expression during host infection, immunohistochemistry experiments were undertaken using our polyclonal anti-FBA-tb antibody and M. tuberculosis-infected lung tissues from IFN-γ/H9253-KO mice and guinea pigs. The location of auramine-rhodamine-positive bacilli in the lungs of mice and guinea pigs over time is detailed in Ref. 28. Representative micrographs of these experiments are presented in Fig. 3. Results unambiguously pointed to the strong expression of FBA-tb by M. tuberculosis bacilli inside the granulomatous lung tissues of IFN-γ/H9253-KO mice (Fig. 3, A and B). Expression of FBA-tb was also clearly detectable in the necrotic core of primary lung granulomas from infected guinea pigs (Fig. 3, C–E). Although the precise physiological state under which the bacilli depicted in Fig. 3, C–E, exist cannot be ascertained, it is noteworthy that persistent bacilli have been shown to reside within such lesions (1, 58). As expected, few bacilli were detected by acid-fast auramine-rhodamine staining in necrotic cores of guinea pigs (Fig. 3, C). However, within these lesions, FBA-tb co-localized with the GroES protein (Rv3418c in M. tuberculosis H37Rv), confirming the association of the protein with M. tuberculosis bacilli (Fig. 3, D and E). Moreover, no signal was observed for the control experiments in which lung sections were treated with the secondary antibody in the absence of the primary antibody. Interestingly, FBA from M. leprae (ML0286c), which shares 87% amino acid identity with FBA-tb, was also detected in subcellular fractions of the leprosy bacillus purified from chronically infected armadillo tissues (supplemental Fig. S2). Thus, consistent with its apparent constitutive expression in both replicating and non-replicating bacteria, FBA-tb is a promising target for the development of anti-M. tuberculosis therapeutics.
non-replicating bacilli in vitro (see previous section) and requirement for growth under glycolytic and gluconeogenic conditions, FBA-tb is also actively produced during host infection.

**FBA-tb Binds to Human Plasminogen**—Proteomics analysis had identified FBA-tb among *M. tuberculosis* H37Rv candidate culture filtrate proteins with human Plg binding capabilities (29). ELISAs with the recombinant native FBA-tb protein confirmed that the protein bound human Plg with an apparent *K*_d*_d* of 6.7 ± 3 nM (Fig. 4A). Binding was inhibited by the lysine analog e-aminoacaproic acid (Fig. 4B) but was not affected by the addition of a large excess of a competitive inhibitor of FBA-tb (TD3; IC50 ~ 4 nM; see Ref. 33 and below) to the reaction mixture (*K*_d*_d* of 6.8 nM in the presence of 0.7 μM TD3). Thus, binding is predominantly mediated by the lysine binding sites of Plg and lysine residues within the FBA-tb protein and is not dependent on the catalytic activity of FBA-tb. In a solution assay (data not shown) or in the presence of fibrin matrices (Fig. 4C), FBA-tb-bound Plg was activated to plasmin by human tPA, but FBA-tb by itself did not activate Plg. In contrast, streptokinase, a bacterial protein known to activate Plg, displayed potent Plg activating activity in the absence of tPA in the reaction mixture (Fig. 4C). Importantly, the ability of FBA-tb-bound plasmin to respond to regulation by the host serpin α2-antiplasmin in the fibrin matrix assay was significantly decreased compared with plasmin incubated either with no additional protein or in the presence of a control protein that does not bind plasmin(ogen) such as BSA (Fig. 4, B–D). Whereas α2-antiplasmin inhibited plasmin activity by 78–88% in the fibrin matrix wells containing Plg + tPA or Plg + tPA + BSA, the percentage of inhibition in the presence of Plg + tPA + FBA-tb was only 42%. Under the conditions of this assay, the decreased response of FBA-tb-bound plasmin to α2-antiplasmin regulation was similar to that observed for streptokinase-plasmin complexes (45.4% inhibition of plasmin activity) (Fig. 4D). Our findings, which support an involvement of the Lys binding sites of the kringle domains of plasmin(ogen) in the attachment of this host molecule to FBA-tb (Fig. 4B), and the fact that the same domains are known to mediate α2-antiplasmin/plasmin interactions (59) suggest that the inhibition of plasmin regulation by α2-antiplasmin in the presence of FBA-tb results from a competitive mechanism between the two proteins for the same binding sites on plasmin.

Despite lacking identifiable secretion signals, a number of glycolytic enzymes including FBP aldolases, glyceraldehyde-3-phosphate dehydrogenases, and enolases have been found to exhibit non-glycolytic functions at the cell surface of several bacterial pathogens contributing to tissue invasiveness and dissemination (40, 60). In the context of TB infection, a deregulation of plasmin-dependent pathways could have a major impact on the inflammatory response and induction of host metalloproteinases with consequences on granuloma formation (61), lung tissue destruction (62), and bacterial dissemination. Experiments using cellular models of infection and *in vivo* studies involving conditional *fba*-tb gene silencing in *M. tuberculosis* H37Rv (63) are in progress to directly test these hypotheses and assess the essential character of FBA-tb throughout the different stages of the infection.
The subunit corresponding to high occupancy binding (0.6) by TD3 is shown in the top panel, whereas that of low occupancy binding (0.3) is shown in the middle panel. P1 and P7 phosphates of TD3 are identified. Residual electron density at the P1 and P7 phosphates binding loci was refined as sulfate ions that are shown in green. Side chains of the histidine residues chelating the catalytic zinc ion are also depicted. The figure was drawn and the superimposition of FBA-tb subunits binding the TD3 inhibitor. The TD3 binding locus is identical in the two subunits. The inhibitor bound in the high occupancy subunit is shown in blue, and it is shown in orange for the low occupancy subunit. In the subunit corresponding to high TD3 occupancy (0.6), the P7 oxyanion participates in three well-defined hydrogen bonding interactions with water molecules that are not present in the subunit exhibiting lower TD3 occupancy (0.3). The presence of solvating water molecules in the high occupancy subunit and their absence in the other subunits were corroborated by electron density omit maps (data not shown). The same binding geometry corresponding to the high occupancy TD3 site was also observed for zinc ion chelating FBP in FBA-tb (19). The extensive superimposition of FBP and TD3 molecules in the FBA-tb active site shown in supplemental Fig. S4 reinforces the role of TD3 as a competitive inhibitor. Binding by TD3, however, does not induce any additional conformational changes with respect to active site binding by FBP.

**FIGURE 5. Binding by substrate analog TD3 in FBA-tb subunits.** A, difference electron density ($F_o - F_c$) simulated annealed omit map showing fit to the electron density map by inhibitor TD3 bound in the active site of FBA-tb. The subunit corresponding to high occupancy binding (0.6) by TD3 is shown in the top panel, whereas that of low occupancy binding (0.3) is shown in the middle panel. P1 and P7 phosphates of TD3 are identified. Residual electron density at the P1 and P7 phosphates binding loci was refined as sulfate ions that are shown in green. Side chains of the histidine residues chelating the catalytic zinc ion are also depicted. The figure was drawn and the superimposition was prepared using the program PyMOL. The catalytic zinc ion is shown as a gray sphere in all figures. TD3 binding further activates sodium ion binding with the P1 oxyanion. The arrows point to the C5-C6 bond in TD3 that corresponds to the weakest region of electron density in the difference map for TD3 in both subunits. The electron density of the difference omit, which is shown outlining the active binding event, was set to the 2σ level instead of 4σ to reflect the partial occupancy by TD3 and metal ion in the active site. B, superimposition of FBA-tb subunits binding the TD3 inhibitor. The TD3 binding locus is identical in the two subunits. The inhibitor bound in the high occupancy subunit is shown in blue, and it is shown in orange for the low occupancy subunit. In the subunit corresponding to high TD3 occupancy (0.6), the P7 oxyanion participates in three well-defined hydrogen bonding interactions with water molecules that are not present in the subunit exhibiting lower TD3 occupancy (0.3). The presence of solvating water molecules in the high occupancy subunit and their absence in the other subunits were corroborated by electron density omit maps (data not shown). The same binding geometry corresponding to the high occupancy TD3 site was also observed for zinc ion chelating FBP in FBA-tb (19). The extensive superimposition of FBP and TD3 molecules in the FBA-tb active site shown in supplemental Fig. S4 reinforces the role of TD3 as a competitive inhibitor. Binding by TD3, however, does not induce any additional conformational changes with respect to active site binding by FBP.
between identical FBP atoms). The high quality of the in silico predictions substantiates the crystallographically determined binding site for TD3 in the liganded FBA-tb crystal structure. Further comparison of the structures of the ligand-bound complexes with that of the native enzyme indicated similar folding of the polypeptide sequence in each subunit of the enzyme regardless of ligand occupancy in the active site and no additional conformational changes upon either TD3 or FBP binding (root mean square deviations, 0.383 and 0.324 Å, respectively).

A distinguishing feature of the FBA-tb-TD3 complex was the substoichiometric binding of TD3 and Zn$^{2+}$ to the enzyme. Subunit occupancies of TD3 and Zn$^{2+}$ indicated limited binding in one homodimer and undetectable levels in the remaining homodimer. Relative levels of TD3 and Zn$^{2+}$ occupancy amounted to an approximate 1:1 stoichiometric ratio in each of the bound subunits. In the active site of the native FBA-tb structure, electron density corresponding to the catalytic Zn$^{2+}$ ion was not detected. This unexpected finding is in contrast to the previous report of the substoichiometric presence of zinc ion in a native form of FBA-tb (0.5 zinc ion per protomer) purified from E. coli (18) and is most likely a consequence of competitive zinc ion sequestration by sulfate ions present in the crystallization buffer (67). A substoichiometric zinc content per subunit in class II FBP aldolases has recently been documented (68) and is as low as 0.16 zinc ion per protomer in the case of FBA from Pseudomonas aeruginosa. Active site binding by the TD3 ligand thus entails a previously unknown reaction mechanism associated with class II aldolases involving apparent stoichiometric recruitment of the catalytic zinc ion by TD3 (a consequence of the chelating properties of the hydroxamate moiety in this compound). Further supporting this reaction mechanism, the same FBP-zinc ion stoichiometry was observed in the structure of the FBA-tb-FBP complex (19), suggesting that ligand-activated Zn$^{2+}$ binding may be a general recruitment mechanism to maximize binding and catalytic activity. Such ligand-activated Zn$^{2+}$ binding has been reported for metallo-β-lactamases where the apo form is the prevailing state under physiological conditions in the absence of substrates (69). Substrate availability apparently induces a spontaneous self-activation due to a decrease of the dissociation constants, resulting in the formation of fully active enzyme. Serendipitously, the crystallization conditions used in this study that rely on molar concentrations of sulfate ion in the crystallization buffer responsible for competitive zinc ion sequestration (67) have provided the first structural corroboration for this observation. For the same reason, the crystallization conditions probably mitigated maximal TD3 activated zinc binding in the crystal lattice, resulting in only 0.9 active sites being occupied of four possible sites.

In addition to competitive sequestration of Zn$^{2+}$, sulfate ions at molar concentration also compete for the phosphate binding sites, thereby inhibiting active site binding, consistent with the complete loss of enzymatic activity observed in the crystallization buffer. The absence of catalytic Zn$^{2+}$ and TD3 binding in one homodimer may thus reflect tighter active site binding by sulfate ions due to differential lattice packing between the homodimers. Enhanced stabilization of the sulfate ion by merely 1 kcal mol$^{-1}$ in this homodimer would effectively diminish the observed Zn$^{2+}$- and TD3-bound population to an occupancy of <0.1, which would not be detectable by structural analysis.

Conclusions—Because of their central involvement in glycolysis, gluconeogenesis, and ATP synthesis under low oxygen tension, fructose-1,6-bisphosphate aldolases represent attractive targets for the development of novel drugs. Accordingly, recent studies have exploited the fact that glycolysis serves as the major source of ATP in some human parasitic protozoans to explore the potential of class I and class II FBAs as therapeutic targets in Giardia lamblia (class II FBA) and in Trypanosoma brucei, Leishmania mexicana, and Plasmodium falciparum (class I FBAs) (66, 70). Despite their widespread distribution in bacteria (including most major bacterial pathogens) and absence from mammalian cells, prokaryotic class II FBAs have been much less studied. The results of our study clearly highlight the potential of the class II fructose-1,6-bisphosphate aldolase of M. tuberculosis as a therapeutic target both from the perspective of its apparent constitutive expres-
sion and requirement for growth and for its potential involvement in the immunopathology of the disease. The lack of effect of a potent substrate analog of FBA-tb on the binding of this protein to Plg alludes to two spatially distinct binding loci that may be useful for target-based drug design. Complementary to our efforts to precisely define the role(s) of FBA-tb during host infection, work is in progress to identify drug-like inhibitors of this promising target.

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