Supporting Information

Eoh and Rhee 10.1073/pnas.1400390111

SI Materials and Methods

Generation of Recombinant Mtb FBPase and Development of in Vitro Enzyme Assay. *glpX* (*Rv1099c*) encoding Mtb FBPase fused to a Strep II tag was amplified by using the primer set: Forward Primer- cat atg gag ctg gtc cgg gtg acc gag gcc gga and Reverse Primer-gct gag cct att ttt cga act gcg ggt ggc tcc aag cgc tgg gca atg ggt aca cgg cgc tgc t containing NdeI and BlpI restriction enzyme site, respectively (underlined). The PCR products were then digested with NdeI and BlpI, and ligated into pET28a (+) vector (EMD Biosciences) that had been digested with the same enzymes to generate expression construct, pET28a(+)::*glpX*. Transformation of *Escherichia coli*, BL21(DE3) (Novagen), with the expression plasmids afforded recombinant strains, BL21 (DE3) [pET28a(+)::*glpX*]. Cells were grown to an OD_{580} of 0.6, treated with 1 mM isopropyl-β-D-thiogalactopyranoside at 20 °C for 16 h, harvested by centrifugation, resuspended in binding buffer [50 mM 4-morpholine propane sulfonic acid (Mops) at pH 7.9, 1mM $MgCl_2$, 10% (vol/vol) glycerol, and 1 mM β-mercaptoethanol], and disrupted by sonication using a Sanyo Soniprep 150 (Integrated Services, TCP Inc.) on ice. Recombinant proteins carrying Strep-tags were purified by immobilized affinity chromatography by using a Strep Tactin II resin (IBA) and analyzed by SDS/PAGE and Western blot analysis using an anti-Strep tag antibody (IBA). Fractions containing pure recombinant protein were desalted by using PD-10 columns and concentrated using a 5,000 molecular weight cutoff Centricon (Millipore).

The EnzChek Phosphate Assay Kit (Invitrogen) was used to monitor the released P*i* generated by FBPase activity (Fig. S4*A*). Reactions were performed in 96-well microplates (Costar) with a 200-μL reaction volumes containing 50 mM Tris·HCl at pH 7.4, 200 μM 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), 1 mM DTT, 100 μM fructose 1,6-bisphosphate, and 1 U PNP. In all cases, reactions were started by the addition of 0.2 μg of purified FBPase, incubated at room temperature for 30 min, and terminated by the addition of 1 μL of 300 mM EDTA. Inhibitory effects of 2-methylcitrate (2-MC) were measured by adding various concentrations of 2-MC to the reactions. The endpoint absorbance (360 nm) was determined by using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments).

Isotopologue Data Analysis Using Isotope Labeled Carbon Sources. The extent of isotopic labeling for metabolites was determined by dividing the summed peak height ion intensities of all labeled isotopologue species by the ion intensity of both labeled and unlabeled species, expressed in percentage. Label-specific ion counts were corrected for naturally occurring ^{13}C species (i.e., [M+1] and [M+2]). The relative abundance of each isotopically labeled species was determined by dividing the peak height ion intensity of each isotopic form (corrected for naturally occurring ^{13}C species as above) by the summed peak height ion intensity of all labeled species. Ion intensities were converted into molar abundances by using standard curves generated by the addition of chemical standards, and serial dilution of samples to establish the colinearity of ion intensity and molar abundance.

Measurement of Membrane Potential and NAD/NADH Ratio. Mtb cultures were grown in m7H9 with 0.2% dextrose to mid-logarithmic phase and concentrated to an $OD_{580} \sim 1.0$ in fresh m7H9. Mtb cultures were then washed with m7H9 with no carbon source, added 0.2% propionate in the presence or absence of vitamin B_{12} (VB_{12}), and inoculated into 96-well microtiter plates, after which 15 μM $DiOC_2$ was added to the well and incubated for an additional 20 min at room temperature. Cultures were subsequently washed with fresh m7H9 to remove extracellular dye. As a positive control for membrane depolarization, cultures were treated with 5 μM protonophore carbonyl-cyanide 3-chlorophenylhydrazone (cccp) (Invitrogen). DMSO was used as a vehicle control. The assay was performed in black with clear-bottom 96-well plates (Costar) and a SpectraMax M5 spectrofluorimeter (Molecular Devices) was used to measure green fluorescence (488 nm/530 nm) and shifts to red fluorescence (488 nm/610 nm). Membrane potential was measured as a ratio of red fluorescence (which depended on cell size and membrane potential) to green fluorescence (which depended on cell size alone). Also, NAD and NADH concentrations were measured by using a FluroNAD/NADH detection kit (Cell Technology). Metabolism of *Mtb* was rapidly quenching by plunging bacilli in the first solvent in the kit. Each condition was measured in triplicate and each experiment was performed twice.

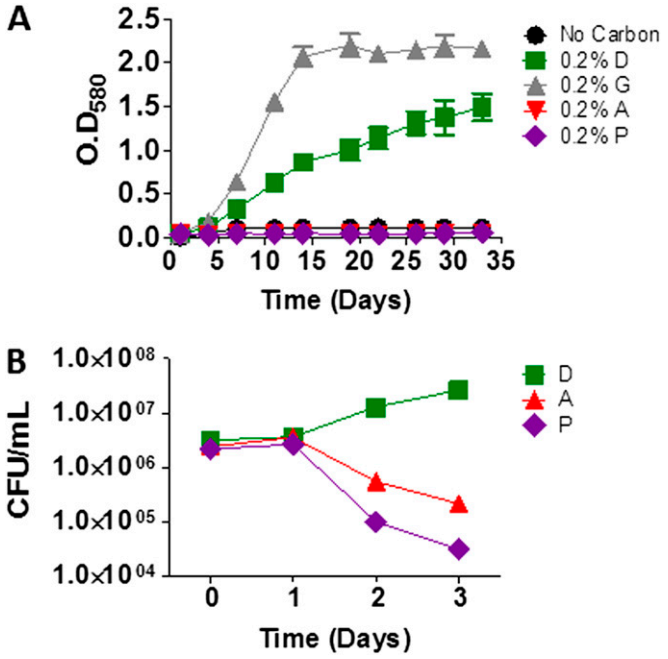

Fig. S1. Bactericidal activity of fatty acid carbon sources to isocitrate lyase (ICL)-deficient *M. tuberculosis*. (*A*) Growth curves of ICL-deficient *M. tuberculosis* cultured with dextrose, acetate, or propionate. (*B*) Colony forming unit (CFU)-based viability of ICL-deficient *M. tuberculosis* cultured with dextrose, acetate, or propionate. All are averages of at least two independent experiments, each of which consisted in biological triplicates, ±SE. A, acetate; D, dextrose; G, glycerol; P, propionate.

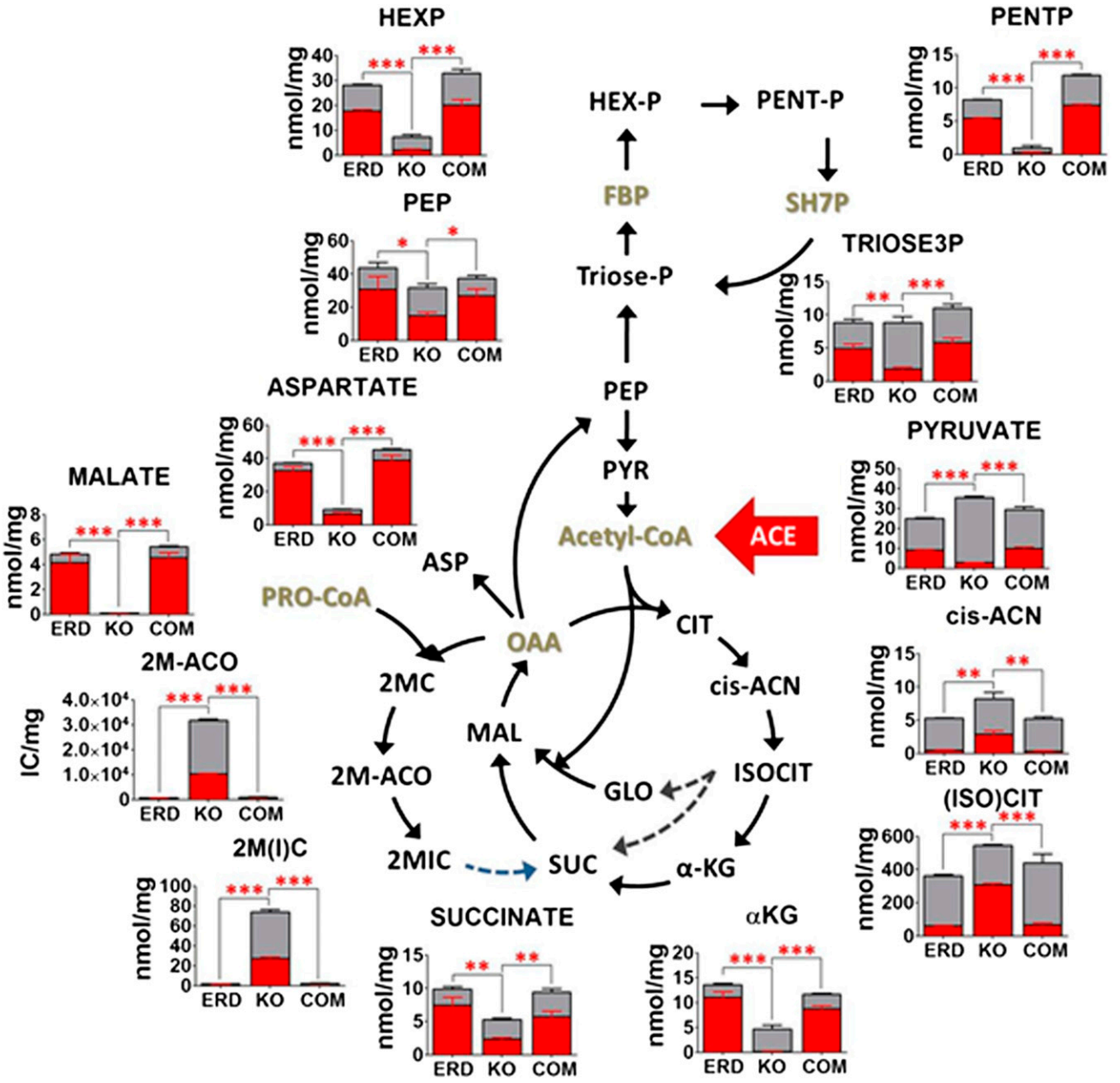

Fig. S2. Metabolomic profile of ICL-deficient *M. tuberculosis* when cultured in media containing acetate. Intrabacterial pool sizes and isotopic labeling of metabolic intermediates of glycolysis/gluconeogenesis, TCA cycle, glyoxylate shunt, and methylcitrate cycle in wild-type (Erdman), ICL1/2-deficient (KO), and ICL-reconstituted (COM) *M. tuberculosis* strains incubated in [U-^{13}C]acetate containing media for 24 h. Total bar heights indicate the intrabacterial concentration, whereas the colored area of each bar denotes the extent of ^{13}C labeling achieved following transfer to [U-^{13}C] acetate (red)-containing media under the condition indicated. ICL encodes both isocitrate lyase (gray dotted line) and methylisocitrate lyase (blue dotted line). All values are the average of three independent experiments, each of which consisted in biological triplicates ± SE. Intrabacterial pool sizes of metabolites analyzed were described as nmol/mg except for 2 methyl cis-aconitate, for which no commercially available authentic compound was available and whose relative abundance is instead reported as ion counts per milligram. ***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$; and ns, not significant by ANOVA. αKG, α-ketoglutarate; 2MC, 2 methylcitrate; 2M-ACO, 2 methyl cis-aconitate; 2MIC, 2 methylisocitrate; Asp, aspartate; cis-ACN, cis-aconitate; FBP, fructose 1, 6-bisphosphate; FUM, fumarate; GLO, glyoxylate; Hex-P, hexose phosphate; IC, ion counts; MAL, malate; Pent-P, pentose 5-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; SH7P, sedoheptulose 7-phosphate; Triose-P, triose 3-phosphate (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate).

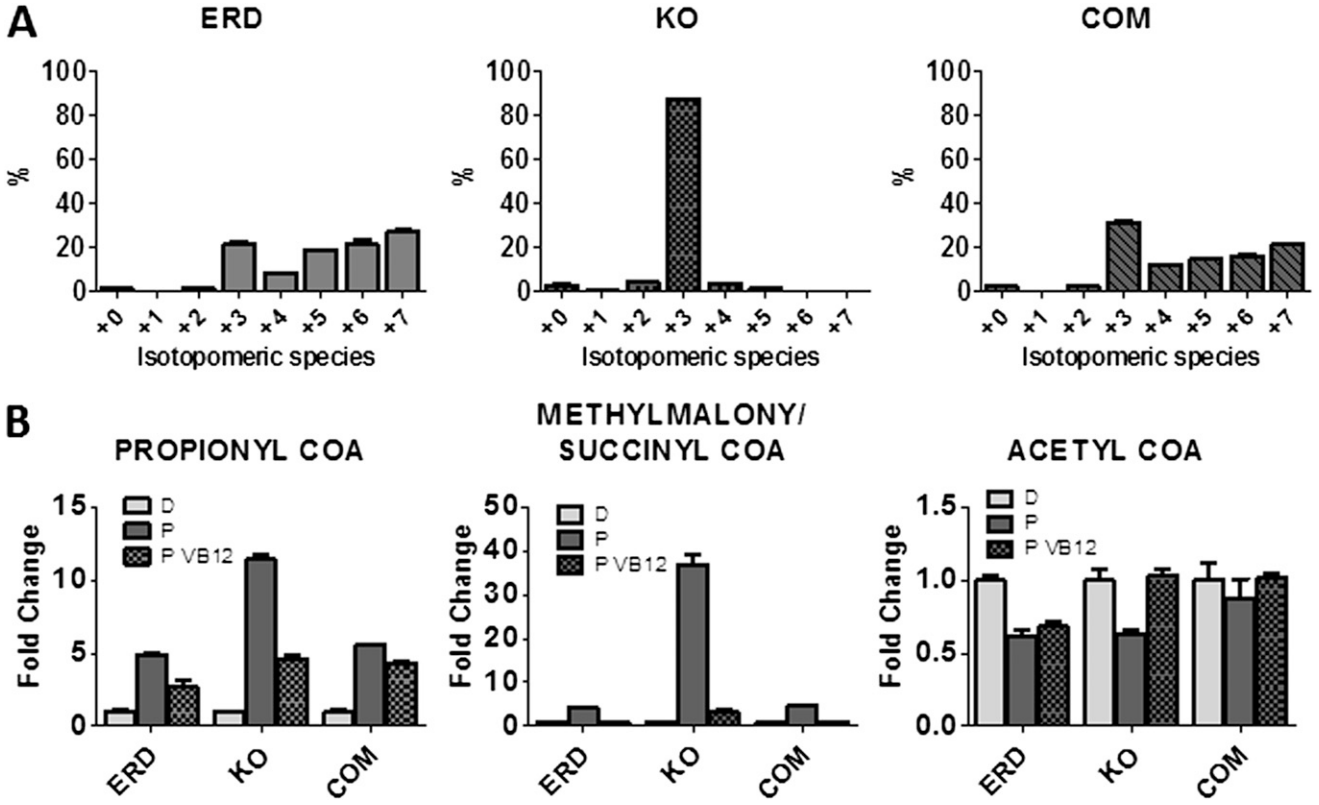

Fig. S3. Metabolic consequences of ICL deficiency in *M. tuberculosis*. (*A*) Percentage isotopologue composition of 2-methyl(iso)citrate when the three strains Erdman WT (ERD), ICL-deficient (KO), and ICL-reconstituted (COM) *M. tuberculosis* strains were cultured in media containing [U-^{13}C]propionate. (*B*) Intrabacterial pool sizes of propionyl-CoA, methylmalonyl/succinyl-CoA, and acetyl-CoA in ERD, KO, and COM when cultured in media containing propionate (P) and comparison with those when cultured in media containing dextrose (D) or propionate in the presence of 10 μg/mL VB_{12} (P VB12). All values are averages of at least two independent experiments, each of which consisted in biological triplicates, ±SE.

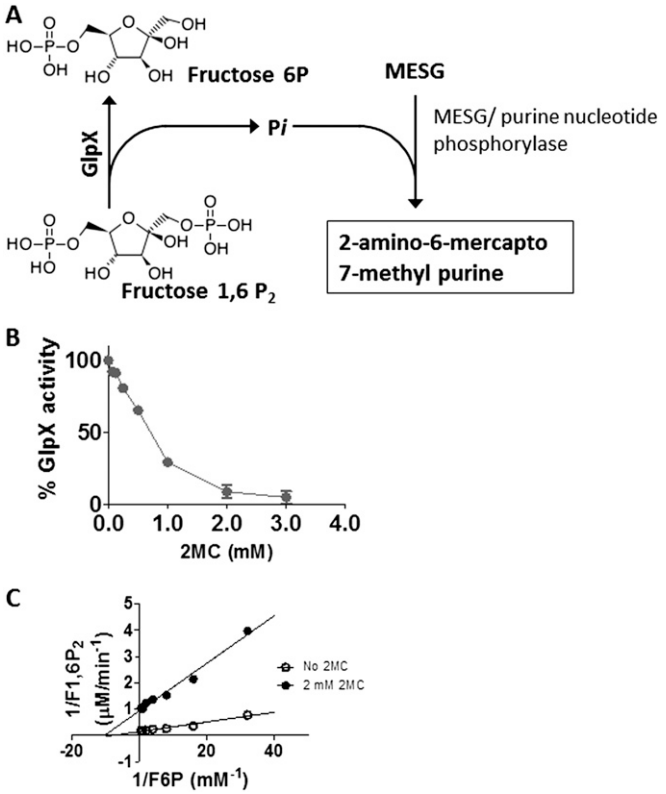

Fig. S4. In vitro inhibition of *M. tuberculosis* Fructose 1,6-bisphosphatase (FBPase, GlpX) by 2-methylcitrate (2MC). (*A*) In vitro spectrophotometric GlpX assay. GlpX produces inorganic phosphate (P*i*), which is used by purine nucleotide phosphorylase to generate 2-amino-6-mercapto-7-methyl purine (highlighted by box) from MESG, that can be detected by absorbance at 360 nm. (*B*) In vitro inhibition of *M. tuberculosis* GlpX activity by varying concentrations of 2MC. (*C*) Double reciprocal (Lineweaver-Burk) plot of *M. tuberculosis* GlpX in the presence or absence of 2 mM 2MC. Reaction conditions are described in text. 2MC deceased v_{max} and had no effect on K_m of *M. tuberculosis* GlpX. $F1,6P_2$, fructose 1,6 bisphosphate; F6P, fructose 6-phosphate.

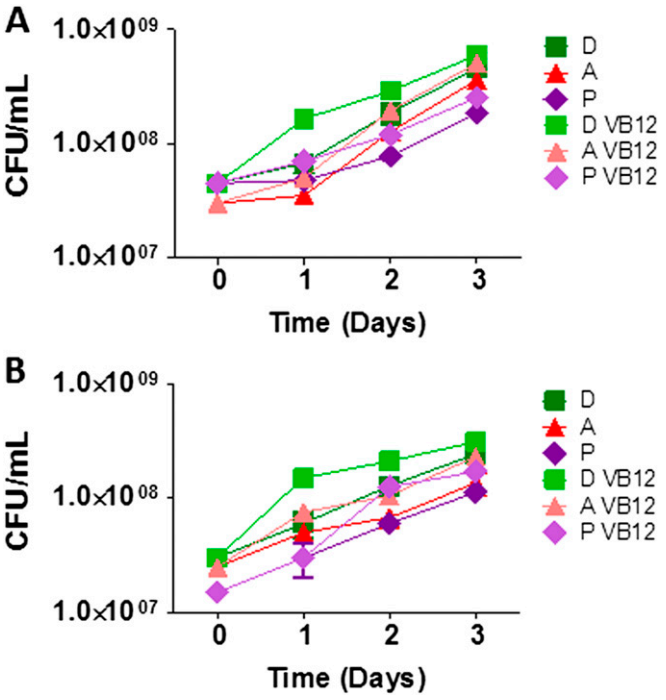

Fig. S5. Chemical-phenotypes Erdman WT (ERD) and ICL-reconstituted (COM) *M. tuberculosis* strains. Growth phenotypes of ERD (*A*) and COM (*B*) in the presence of various carbon substrates in the presence or absence of VB_{12}. A, acetate; D, dextrose; P, propionate; VB12, 10 μg/mL VB_{12}. All are averages of at least two independent experiments, each of which consisted in biological triplicates, ±SE.

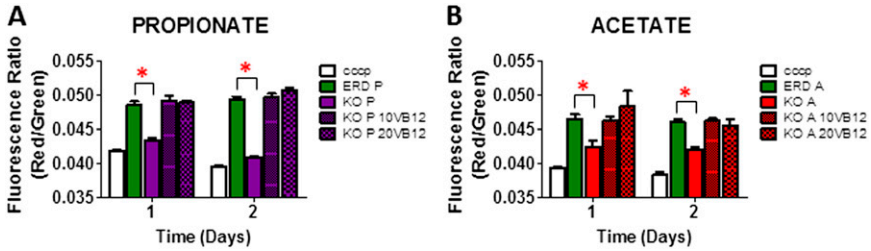

Fig. S6. Biochemical characterization of *M. tuberculosis* cultured on various carbon sources and the impact of genetic ICL deficiency. Depolarized membrane potential of ICL-deficient *M. tuberculosis* cultured in the presence of either propionate (*A*) or acetate (*B*) at 1 or 2 d incubation and its restoration by addition of varying amounts (10 or 20 μg/mL) of VB_{12}. Cultures grown on media containing 5 μM protonophore carbonyl-cyanide 3-chlorophenylhydrazone (cccp), a membrane depolarization agent, were used as a positive control and those with DMSO (ERD P or ERD A) were as a vehicle control. 10 or 20VB12, 10 or 20 μg/mL VB_{12}; A, acetate; ITA, itaconic acid; P, propionate. All are averages of at least two independent experiments, each of which consisted in biological triplicates, ±SE. *$P < 0.001$ by ANOVA.

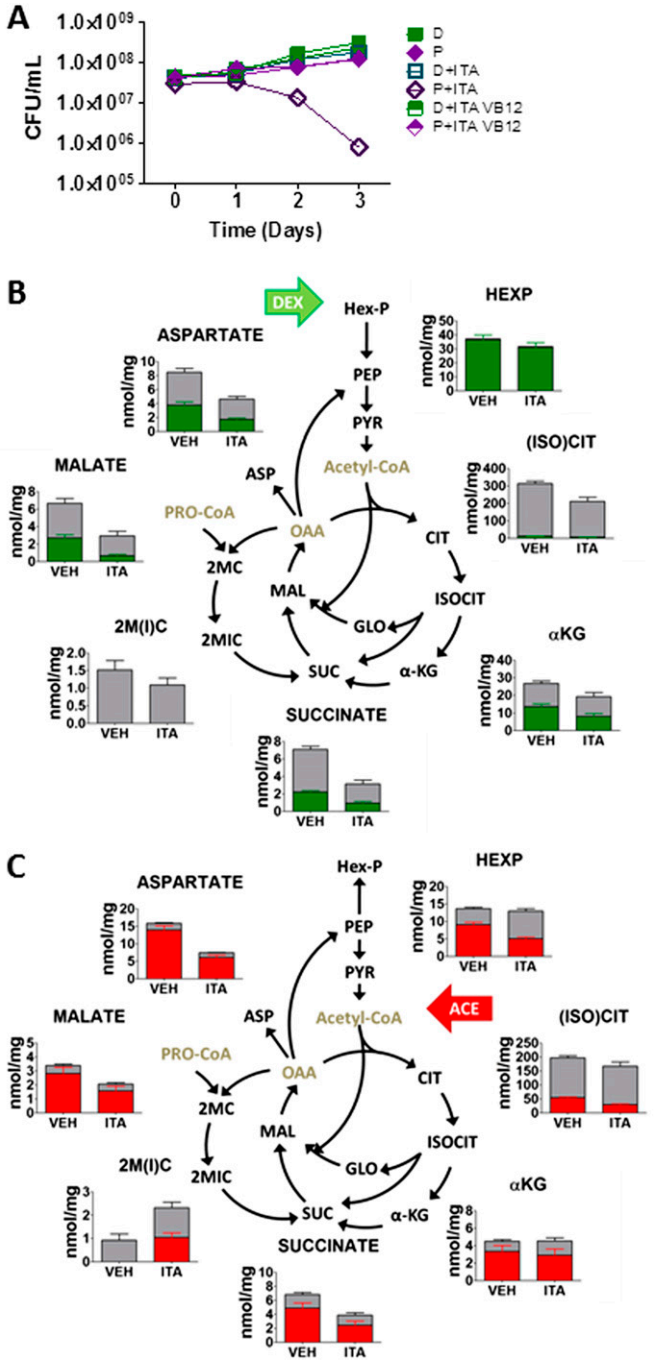

Fig. S7. The effect of 2 mM itaconic acid (ITA) associated ICL inhibition on *M. tuberculosis* viability. (*A*) Viability was reported by colony forming unit (CFU) of *M. tuberculosis* $H_{37}Rv$ cultured with dextrose (D) or propionate (P) in the presence or absence of 2 mM itaconic acid. The propionate and itaconic acid combined bactericidal effect was alleviated by addition of 10 μg/mL VB12. (*B* and *C*) Metabolic effect of 2 mM ITA treatment on *M. tuberculosis* $H_{37}Rv$ central carbon metabolism when cultured in the presence of dextrose (*B*) or acetate (*C*) as single carbon sources. All are averages of at least two independent experiments, each of which consisted in biological triplicates, ±SE. All abbreviations are followed as in Fig. 1 or Fig. S2.

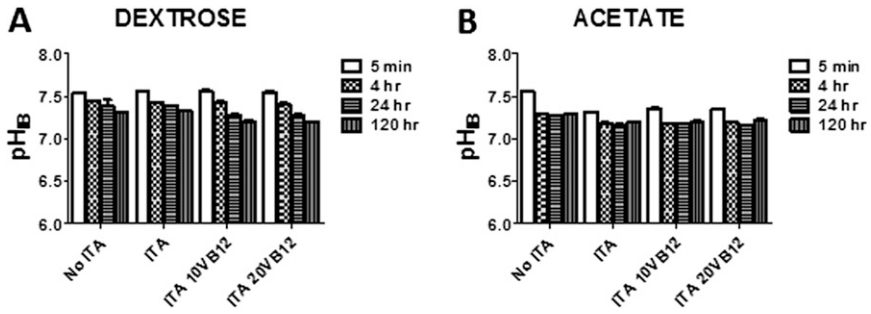

Fig. S8. Biochemical characterization of *M. tuberculosis* cultured on various carbon sources and the impact of chemical-ICL deficiency. Determination of intrabacterial pH of *M. tuberculosis* H_{37}Rv cultured in the presence of dextrose (*A*) or acetate (*B*) with or without 2 mM itaconic acid and either 10 or 20 μg/mL VB_{12} in a time-dependent manner. All are averages of at least two independent experiments, each of which consisted in biological triplicates, ±SE.