Microbiology (2007), **153**, 3973–3982 DOI 10.1099/mic.0.2007/011726-0

Role of the methylcitrate cycle in propionate metabolism and detoxification in *Mycobacterium smegmatis*

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Received 16 July 2007
Revised 6 September 2007
Accepted 10 September 2007

Catabolism of odd-chain-length fatty acids yields acetyl-CoA and propionyl-CoA. A common pathway of propionyl-CoA metabolism in micro-organisms is the methylcitrate cycle, which includes the dedicated enzymes methylcitrate synthase (MCS), methylcitrate dehydratase (MCD) and methylisocitrate lyase (MCL). The methylcitrate cycle is essential for propionate metabolism in *Mycobacterium tuberculosis*. Unusually, *M. tuberculosis* lacks an MCL orthologue and this activity is provided instead by two isoforms of the glyoxylate cycle enzyme isocitrate lyase (ICL1 and ICL2). These bifunctional (ICL/MCL) enzymes are jointly required for propionate metabolism and for growth and survival in mice. In contrast, the non-pathogenic species *Mycobacterium smegmatis* encodes a canonical MCL enzyme in addition to ICL1 and ICL2. The *M. smegmatis* gene encoding MCL (*prpB*) is clustered with genes encoding MCS (*prpC*) and MCD (*prpD*). Here we show that deletion of the *M. smegmatis prpDBC* locus reduced but did not eliminate MCL activity in cell-free extracts. The residual MCL activity was abolished by deletion of *icl1* and *icl2* in the Δ*prpDBC* background, suggesting that these genes encode bifunctional ICL/MCL enzymes. A Δ*prpB* Δ*icl1* Δ*icl2* mutant was unable to grow on propionate or mixtures of propionate and glucose. We hypothesize that incomplete propionyl-CoA metabolism might cause toxic metabolites to accumulate. Consistent with this idea, deletion of *prpC* and *prpD* in the Δ*prpB* Δ*icl1* Δ*icl2* background paradoxically restored growth on propionate-containing media. These observations suggest that the marked attenuation of ICL1/ICL2-deficient *M. tuberculosis* in mice could be due to the accumulation of toxic propionyl-CoA metabolites, rather than inability to utilize fatty acids per se.

INTRODUCTION

Accumulating evidence suggests an important role for fatty acid catabolism in mycobacteria and other microbial pathogens during infection (Boshoff & Barry, 2005; Munoz-Elias & McKinney, 2006). Consistent with this idea, the anaplerotic glyoxylate cycle, which includes the enzymes isocitrate lyase (ICL) and malate synthase (MLS) (Fig. 1b; Table 1), is required for fatty acid metabolism and virulence of diverse bacterial and fungal pathogens (Munoz-Elias & McKinney, 2006). The glyoxylate cycle is required for assimilation of acetyl-CoA units derived from beta-oxidation of fatty acids. Beta-oxidation of odd-chain-length fatty acids yields propionyl-CoA as an additional product. Several microbial pathways of propionyl-CoA metabolism have been proposed (Horswill & Escalante-Semerena, 1999; Textor *et al.*, 1997), including the methylcitrate cycle, which mediates oxidation of propionyl-CoA to pyruvate.

Three enzymes are thought to be specific to the methylcitrate cycle: methylcitrate synthase (MCS), methylcitrate dehydratase (MCD) and methylisocitrate lyase (MCL), encoded by the *prpC, prpD* and *prpB* genes, respectively (Fig. 1a; Table 1) (Bramer & Steinbuchel, 2001; Bramer *et al.*, 2002; Brock *et al.*, 2000, 2001; Claes *et al.*, 2002; Horswill & Escalante-Semerena, 1999; Textor *et al.*, 1997). Species that operate both the glyoxylate cycle and methylcitrate cycle typically produce dedicated ICL and MCL enzymes with unique substrate specificities (isocitrate and methylisocitrate, respectively) and non-overlapping roles in their respective pathways (Bramer & Steinbuchel, 2001; Brock *et al.*, 2001; Brock, 2005; Claes *et al.*, 2002; Horswill & Escalante-Semerena, 1999; Liu *et al.*, 2005; Luttik *et al.*, 2000). The active sites of these enzymes also have characteristic catalytic motifs: K[K/Q]CGH in ICL and KRCGH in MCL (Brock *et al.*, 2001; Brock, 2005; Grimek *et al.*, 2003; Grimm *et al.*, 2003).

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Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoate); ICL, isocitrate lyase; LDH, lactate dehydrogenase; MCD, methylcitrate dehydratase; MCL, methylisocitrate lyase; MCS, methylcitrate synthase; MLS, malate synthase; PBST, PBS containing 0.05 % Tween-80.

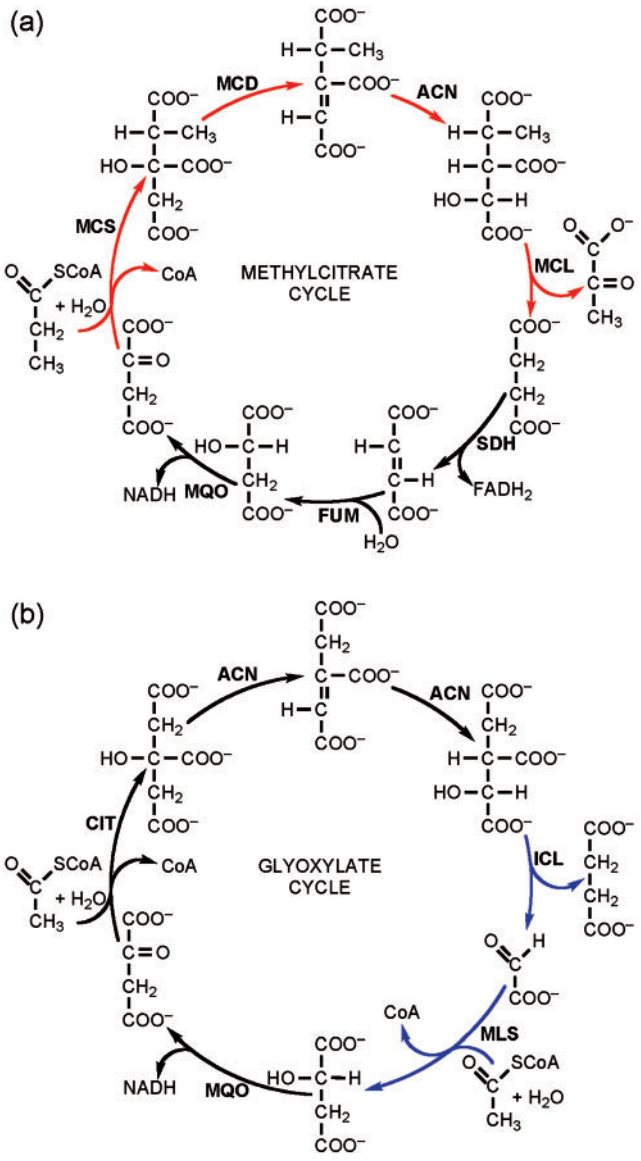

Fig. 1. The methylcitrate and glyoxylate cycles in *Mycobacterium smegmatis*. (a and b) Odd-chain-length fatty acids are degraded by the beta-oxidation cycle to propionyl-CoA and acetyl-CoA units, which are further metabolized by the methylcitrate cycle and the glyoxylate cycle, respectively. The methylcitrate cycle (a) converts propionyl-CoA to pyruvate on an equimolar basis. The glyoxylate cycle (b) converts two molar equivalents of acetyl-CoA to one molar equivalent of succinate. Enzymes: MCS (*prpC*), methylcitrate synthase; MCD (*prpD*), methylcitrate dehydratase; ACN, aconitase; MCL (*prpB*), methylisocitrate lyase; SDH, succinate dehydrogenase; FUM, fumarase; MQO, malate:quinone oxidoreductase; CIT, citrate synthase; ICL (*icl1*, *icl2*), isocitrate lyase; MLS (*glcB*), malate synthase. Reactions unique to the methylcitrate cycle (red arrows) or the glyoxylate cycle (blue arrows) are indicated. All other reactions (black arrows) also occur in the citric acid cycle.

The *Mycobacterium tuberculosis* and *Mycobacterium bovis* genomes encode MCD (*prpD*; *mt1162*; *mb1161*) and MCS (*prpC*; *mt1163*; *mb1162*) orthologues but do not encode an MCL (*prpB*) orthologue (Cole *et al.*, 1998; Fleischmann *et al.*, 2002; Garnier *et al.*, 2003), and all three enzymes are apparently absent in *Mycobacterium leprae* (Cole *et al.*, 2001), suggesting the absence of a functional methylcitrate cycle in these species. However, we found that *M. tuberculosis* requires the *prpDC* genes for *in vitro* metabolism of propionate and other odd-chain-length fatty acids, implying that the methylcitrate cycle is intact (Munoz-Elias *et al.*, 2006). In contrast to other species, where ICL cannot substitute for MCL in propionate metabolism, the *M. tuberculosis* ICL1 (*mt0483*) and ICL2 (*mt1966*) orthologues appear to be bifunctional ICL/MCL enzymes that participate in both the glyoxylate cycle and the methylcitrate cycle, despite possessing the canonical KKCGH motif that is characteristic of monofunctional ICL (Gould *et al.*, 2006; Munoz-Elias *et al.*, 2006). The recently solved three-dimensional structures of ICL1 bound with the MCL substrate methylisocitrate or with the MCL reaction products (succinate and pyruvate) demonstrate that the active site of this enzyme can accommodate these moieties and catalyse the MCL reaction (Gould *et al.*, 2006).

Consistent with the idea that ICL1/ICL2 perform essential functions in both the glyoxylate cycle and the methylcitrate cycle, these enzymes are jointly required for growth of *M. tuberculosis* on even- or odd-chain-length fatty acids (including acetate and propionate), and their activity is acetate- and propionate-inducible (Munoz-Elias & McKinney, 2005; Munoz-Elias *et al.*, 2006; unpublished observations). However, in some micro-organisms the glyoxylate cycle is required for carbon anaplerosis during growth on propionate, even when propionate is metabolized by pathways other than the methylcitrate cycle (Ashworth & Kornberg, 1964; Bramer & Steinbuchel, 2001; Wang *et al.*, 2003; Wegener *et al.*, 1969). Thus, it is unclear whether the essential role of ICL1/ICL2 in growth and survival of *M. tuberculosis* during infection (Munoz-Elias & McKinney, 2005) is due to their participation in the glyoxylate cycle, the methylcitrate cycle, or both pathways. Interpretation of these observations is further complicated by studies in *Aspergillus nidulans* (Brock, 2005) and *Salmonella typhimurium* (Horswill *et al.*, 2001), which revealed that the methylcitrate cycle intermediates methylcitrate and methyl-*cis*-aconitate – generated by MCS and MCD, respectively – are growth-inhibitory in these species. These intermediates would be expected to accumulate in the absence of MCL activity, and could account for the severe attenuation of ICL1/ICL2-deficient *M. tuberculosis in vivo*.

In order to elucidate the roles of the glyoxylate and methylcitrate cycles in mycobacterial metabolism, we carried out studies in *Mycobacterium smegmatis*, a fast-growing relative of *M. tuberculosis* that is more amenable to genetic and biochemical analysis. In addition to ICL1 (*msm0911*) and ICL2 (*msm3706*) orthologues, the *M.*

Table 1. Putative glyoxylate and methylcitrate cycle enzymes and genes in *M. smegmatis*, *M. tuberculosis* and *C. glutamicum*

Protein (*gene*) ID	*M. smegmatis**		*M. tuberculosis**			*C. glutamicum**		
	Locus	Length	Locus†	Length	Identity (%)‡	Locus§	Length	Identity (%)‡
MCL (*prpB*)	*msm6646*	305	–	–	–	*cg0760*	307	64.8
ICL1 (*icl1*)	*msm0911*	428	*mt0483*	428	92.1	*cg2560*	432	81.7
ICL2 (*icl2*)	*msm3706*	769	*mt1966*	766	77.1	–	–	–
MLS (*glcB*)	*msm3640*	732	*mt1885*	741	78.4	*cg2559*	739	61.4
CIT1 (*gltA*)	*msm5672*	434	*mt0920*	431	84.1	*cg0949*	437	63.9
CIT2 (*citA*)	*msm5676*	377	*mt0912*	373	85.7	–	–	–
MCS (*prpC*)	*msm6647*	376	*mt1163*	393	75.9	*cg0762*	383	54.4
MCD (*prpD*)	*msm6645*	460	*mt1162*	526	71.5	*cg0759*	504	66.1

*Annotated genomes can be accessed at http://www.tigr.org.

†The *mt1162-mt1163* locus is required for propionate metabolism in *M. tuberculosis* (Munoz-Elias *et al.*, 2006).

‡Comparisons were made between the predicted polypeptide sequences using Vector NTI 6.0 AlignX, which uses the CLUSTAL W algorithm (Thompson *et al.*, 1994). Values indicate the percentage amino acid identity between the *M. smegmatis* polypeptide and its orthologues in *M. tuberculosis* or *C. glutamicum*.

§The *cg0759-cg0762* locus is required for propionate metabolism in *C. glutamicum* (Kalinowski *et al.*, 2003). A paralogous gene cluster – comprising *cg0797* (*prpB1*), *cg0798* (*prpC1*) and *cg0796* (*prpD2*) – appears to be cryptic (non-functional) in this species under standard growth conditions (Kalinowski *et al.*, 2003).

smegmatis genome potentially encodes a dedicated MCL (*prpB*; *msm6646*), which appears to be arranged in an operon (*prpDBC*) encoding MCS (*prpC*; *msm6647*) and MCD (*prpD*; *msm6645*) orthologues. Similarly arranged orthologues of *prpDBC* are present in the *Mycobacterium avium* (*mav0346-mav0345-mav0344*) and *Mycobacterium marinum* (*mmar1379-mmar1380-mmar1381*) genomes (Li *et al.*, 2005; annotated *M. marinum* genome available at http://genolist.pasteur.fr/MarinoList/). Here, we evaluate the role of the methylcitrate cycle and the contribution of the *prpDBC* and *icl1*/*icl2* gene products to propionate metabolism in *M. smegmatis*. We provide evidence supporting the idea that the accumulation of toxic metabolites is growth-inhibitory to ICL/MCL-deficient bacteria during growth on propionate-containing media. These observations suggest that the essential role of ICL1/ICL2 in *M. tuberculosis* metabolism during infection might be in propionate detoxification rather than (or in addition to) their role in fatty acid catabolism.

METHODS

Bacteriology. *M. smegmatis* strain mc^{2}155 (Snapper *et al.*, 1990) and derivative strains were stored at −80 °C in 15 % glycerol. Bacteria were grown with aeration at 37 °C in Middlebrook 7H9 (DIFCO) broth containing 0.5 % BSA fraction V (Fisher), 0.085 % NaCl, 0.05 % Tween-80 and 0.2 % glucose (Sigma), or on Middlebrook 7H10 (DIFCO) agar containing 10 % oleic-albumin-dextrose-catalase (DIFCO) and 0.5 % glycerol. For carbon utilization experiments and preparation of cell-free extracts, bacteria were grown in M9 broth [M9 salts (DIFCO), 0.1 mM $CaCl_2$, 2 mM $MgSO_4$ (Sigma)], containing the indicated carbon substrate at 0.1 % or 0.5 % (w/v). Antibiotics (Sigma) were kanamycin (25 μg ml^{-1}), hygromycin (50 μg ml^{-1}) and streptomycin (20 μg ml^{-1}). Culture turbidity (OD_{600}) measurements were made using an Ultrospec 2000 spectrophotometer (Pharmacia) following dilution with PBS containing 0.05 % Tween-80 (PBST), to give readings within the range 0.05–0.25.

M. smegmatis* cell-free extracts.** Bacteria were grown to late exponential phase (OD_{600} 1.0–1.3) in M9 broth containing the indicated carbon substrate, collected by centrifugation (5000 ***g, 20 min), washed thrice with PBST and resuspended in the appropriate assay buffer supplemented (5 % v/v) with protease inhibitor cocktail (Sigma P-8465). Cells were disrupted by bead-beating (BioSpec) with 0.1 mm zirconia-silica beads (Sigma) for 60 s at high speed. Extracts were clarified by centrifugation (18 000 ***g***, 15 min), and the total protein concentration was determined by Bradford assay (Sigma). Cell-free extracts were stored frozen at −80 °C.

MCS assays. MCS activity was measured by following the accumulation of free CoA, generated by the condensation of propionyl-CoA and oxaloacetate, after reaction of CoA with 5,5′-dithiobis-(2-nitrobenzoate) (DTNB), as described (Munoz-Elias *et al.*, 2006). Reactions were done at room temperature in a 1 ml assay volume containing 50 mM HEPES-NaOH pH 8.0, 0.1 M NaCl, 2 mM EDTA, 0.1 mM DTNB, 0.035 mM propionyl-CoA and 0.4 mM oxaloacetate. Reactions were started by addition of cell-free extract (5–300 μg total protein in 5–50 μl). Oxaloacetate-stimulated TNB anion formation, generated by the reaction of free CoA with DTNB was monitored spectrophotometrically at 412 nm, using an Ultrospec 2000 spectrophotometer (Pharmacia) and the standard extinction coefficient 13.6 mM^{-1} for a 1 cm path length. Background was measured and subtracted by carrying out mock reactions without addition of oxaloacetate. Corrected activities below 1 nmol min^{-1} mg^{-1} were considered to be below the detection limit.

ICL and MCL assays. ICL activity was measured by following the lactate dehydrogenase (LDH)-mediated reduction of glyoxylate to glycolate with concomitant oxidation of NADH, as described

(Munoz-Elias *et al.*, 2006). Reactions were done at RT in a 1 ml assay volume containing 50 mM MOPS-HCl pH 6.8, 5 mM $MgCl_2$, 0.1 mM NADH, 7 U LDH (Roche) and cell-free extract (5–100 μg in 5–100 μl). Reactions were pre-incubated for 5 min and started by addition of 1 mM DL-*threo*-isocitrate (Sigma). Isocitrate-stimulated NADH oxidation was measured spectrophotometrically at 340 nm, using an Ultrospec 2000 spectrophotometer (Pharmacia) and the standard extinction coefficient 6.22 mM^{-1} for a 1 cm path length. MCL activity was measured by following the LDH-mediated reduction of pyruvate to lactate with concomitant oxidation of NADH. 2-Methylisocitrate-stimulated NADH oxidation was measured using the same reaction conditions as described above for measurement of ICL activity, except that reactions contained 2 mM DTT and 1 mM DL-*threo*-2-methylisocitrate (Munoz-Elias *et al.*, 2006) was substituted for DL-*threo*-isocitrate. Background was measured and subtracted by carrying out mock reactions without addition of DL-*threo*-isocitrate (ICL assays) or DL-*threo*-2-methylisocitrate (MCL assays). Corrected activities below 1 nmol min^{-1} mg^{-1} were considered to be below the detection limit.

Construction of Δ*prpB*, Δ*prpB* Δ*icl1* Δ*icl2*, Δ*prpDBC* and Δ*prpDBC* Δ*icl1* Δ*icl2* strains of *M. smegmatis*. In-frame unmarked (non-polar) deletions of the *msm6646* (*prpB*) and *msm6645-6647* (*prpDBC*) ORFs were constructed in the *M. smegmatis* chromosome by two-step (insertion–excision) homologous recombination with the suicide vector pJG1111 (Munoz-Elias *et al.*, 2006) containing *aph* (kanamycin resistance), *hyg* (hygromycin resistance), *sacB* (sucrose sensitivity) and *lacZ* (β-galactosidase) under the control of the *M. tuberculosis* antigen 85 promoter. The Δ*prpB* and Δ*prpDBC* deletions were constructed on the wild-type and Δ*icl1* Δ*icl2* genetic backgrounds. The Δ*icl1* Δ*icl2* strain was generously provided by Ernesto Muñoz-Elías and Lubomir Merkov (The Rockefeller University, New York).

The Δ*prpB* recombination substrate was constructed by PCR amplification of 1 kb regions upstream (fragment A) and downstream (fragment B) of the *prpB* ORF. Primers introduced 5′ *Pac*I and 3′ *Avr*II sites into fragment A and 5′ *Avr*II and 3′ *Asc*I sites into fragment B. Primers for fragment A were 5′-ttaattaagactcggcaccatgc-3′ and 5′-cctaggcatcagcccgct**cat**tgg-3′ (restriction sites are underlined; *prpB* start codon is in bold type). Primers for fragment B were 5′-cctaggagagca**tga**ccacagcaa-3′ and 5′-ggcgcgccatcgggaagtccatcagg-3′ (restriction sites are underlined; *prpB* stop codon is in bold type). The amplicons were digested with *Pac*I and *Avr*II (fragment A) or *Avr*II and *Asc*I (fragment B) and ligated together into the unique *Pac*I and *Asc*I sites of pJG1111 to generate pAU101.

The Δ*prpDBC* recombination substrate was constructed by PCR amplification of 1 kb regions upstream of the *prpD* ORF (fragment C) and downstream of the *prpC* ORF (fragment D). Primers introduced 5′ *Pac*I and 3′ *Avr*II sites into fragment C and 5′ *Avr*II and 3′ *Asc*I sites into fragment D. Primers for fragment C were 5′-ttaattaa-gacgggccagccggtcga-3′ and 5′-cctaggaatacg**cat**gatccgcac-3′ (restriction sites are underlined; *prpD* start codon is in bold type). Primers for fragment D were 5′-cctaggcttgtc**tga**gcgcgcgat-3′ and 5′-ggcgcgcct-ggctcagaccgagatcg-3′ (restriction sites are underlined; *prpC* stop codon is in bold type). The amplicons were digested with *Pac*I and *Avr*II (fragment C) or *Avr*II and *Asc*I (fragment D) and ligated together into the unique *Pac*I and *Asc*I sites of pJG1111 to generate pAU102.

pAU101 (Δ*prpB*) and pAU102 (Δ*prpDBC*) were inserted into the *M. smegmatis* chromosome by electroporation and selection of transformants on 7H10 agar containing hygromycin, kanamycin and X-Gal (50 μg ml^{-1}). Blue colonies were individually picked and amplified in 7H9 broth (no antibiotics) to allow plasmid excision, then plated on 7H10 agar containing X-Gal and 5 % sucrose (no antibiotics) to select for cells in which plasmid excision had occurred. White colonies were individually picked and amplified in 7H9 broth (no antibiotics) for genomic DNA isolation and strains in which the Δ*prpB* or Δ*prpDBC* allele had replaced the corresponding wild-type locus were identified by PCR analysis (not shown). The Δ*prpB* allele is a fused ORF with the sequence **atg**agcgggcagatgcctaggagagca**tga**, comprising the first five and the last two codons of *prpB*, joined by a two-codon linker provided by the introduced *Avr*II site (underlined). The Δ*prpDBC* allele is a fused ORF with the sequence **atg**cgtattcctaggcttgtc**tga**, comprising the first three codons of *prpD* and the last two codons of *prpC* joined by a two-codon linker provided by the introduced *Avr*II site (underlined).

Complementation analysis. The complementing plasmid pPRPDC, containing the *M. tuberculosis prpDC* genes, was described previously (Munoz-Elias *et al.*, 2006). The complementing plasmid pPRPB was constructed by cloning the PCR-amplified *M. smegmatis prpB* ORF into the unique *Hin*dIII site of pEM262, an episomal vector derived from the shuttle vector pMV261 by replacing the *aph* kanamycin resistance cassette with the *aadA* streptomycin resistance cassette (Munoz-Elias & McKinney, 2005). PCR primers, designed to introduce 5′ and 3′ *Hin*dIII sites (underlined) into the *prpB* amplicon, were 5′-aagcttcaatgagcgggctgatgg-3′ and 5′-aagcttgt-catgctctcacctcctg-3′. pPRPB was introduced into *M. smegmatis* by electroporation and selection of transformants on 7H10 agar containing streptomycin.

RESULTS

Bioinformatic analysis of the methylcitrate cycle in *M. smegmatis*

A cluster of three ORFs was identified in the *M. smegmatis* genome (*msm6645-6647*) whose predicted peptide products were highly similar to enzymes of the methylcitrate cycle in *M. tuberculosis* (Munoz-Elias *et al.*, 2006) and the related actinomycete *Corynebacterium glutamicum* (Claes *et al.*, 2002; Kalinowski *et al.*, 2003) (Fig. 1a; Table 1). The *msm6647* ORF encoded a conceptual product of 376 aa with 76 % identity to the *M. tuberculosis prpC* (*mt1163*) gene product and 54 % identity to the *prpC2* (*cg0762*) gene product in *C. glutamicum*. Deletion of the *mt1163* gene in *M. tuberculosis* eliminated all detectable MCS activity in cell-free extracts, thus confirming the assignment of the gene as a functional *prpC* orthologue (Munoz-Elias *et al.*, 2006). The *msm6645* ORF encoded a putative protein (460 aa) with 72 % identity to the *M. tuberculosis prpD* (*mt1162*) gene product and 66 % identity to the *prpD2* (*cg0759*) gene product in *C. glutamicum*. In contrast with *M. tuberculosis*, which lacks a *prpB* orthologue, the *M. smegmatis msm6646* ORF encodes a putative protein (305 aa) with 64 % identity to the *C. glutamicum prpB2* (*cg0760*) gene product.

Role of the *M. smegmatis prpDBC* locus in propionate metabolism

Previously we reported that deletion of the *prpDC* locus eliminated growth of *M. tuberculosis* on media containing propionate as the carbon source (Munoz-Elias & McKinney, 2005). To investigate whether the methylcitrate cycle is also important for propionate metabolism in *M.*

smegmatis, we constructed a strain in which the putative *prpDBC* locus was deleted. The *M. smegmatis prpD*, *prpB* and *prpC* genes appear to be organized in an operon, in which the first and last codons of *prpB* overlap the last and first codons, respectively, of *prpD* and *prpC*. We generated an unmarked, non-polar chromosomal deletion encompassing all three genes, in which codon 3 of *prpD* was fused in-frame to codon 375 of *prpC* (see Methods).

Deletion of *prpDBC* did not alter the kinetics of *M. smegmatis* growth in standard Middlebrook 7H9 broth or in minimal M9 liquid medium supplemented with 0.1 % glucose as the sole carbon source (not shown). However, growth of the Δ*prpDBC* strain was substantially delayed, compared to the parental strain, when the carbon source was 0.1 % (Fig. 2a) or 0.5 % (Fig. 2b) propionate. These observations suggest that, although the *prpDBC* locus contributes to propionate catabolism in *M. smegmatis*, it is not essential. In contrast, the orthologous *prpDC* locus in *M. tuberculosis* is absolutely required for growth on propionate-containing media (Munoz-Elias *et al.*, 2006).

MCS activity in cell-free extracts from wild-type and Δ*prpDBC* bacteria

The delayed but robust growth of Δ*prpDBC* bacteria on propionate suggested the existence of an alternative pathway(s) for propionate metabolism in *M. smegmatis*. To rule out the possibility of a cryptic methylcitrate cycle operating in the absence of *prpDBC*, we confirmed that deletion of *prpDBC* resulted in the loss of MCS activity by assaying cell-free extracts prepared from bacteria grown in media containing glucose or propionate as the sole carbon source. MCS activity was readily detectable in extracts from wild-type bacteria grown on propionate, but was undetectable in extracts from wild-type bacteria grown on glucose or from Δ*prpDBC* bacteria grown on either substrate (Table 2). These results confirm that MCS activity is propionate-inducible in *M. smegmatis*, similar to *M. tuberculosis* (Munoz-Elias *et al.*, 2006), and suggest that *prpC* is the only gene encoding detectable MCS activity in *M. smegmatis*. Apparently, the products of the *msm5672* and *msm5676* ORFs (Table 1), which encode probable type I and type II citrate synthases that are weakly homologous to the predicted *prpC* product (28 % and 26 % identical, respectively), do not possess significant MCS activity. We cannot, however, rule out the possibility that the Δ*prpDBC* strain produces an alternative MCS whose activity is undetectable under the *in vitro* assay conditions that we used.

MCL and ICL activities in cell-free extracts from wild-type and Δ*prpDBC* bacteria

We also measured MCL activity in cell-free extracts prepared from wild-type and Δ*prpDBC* bacteria. MCL activity was barely detectable in cell-free extracts from either strain when cells were grown in media containing

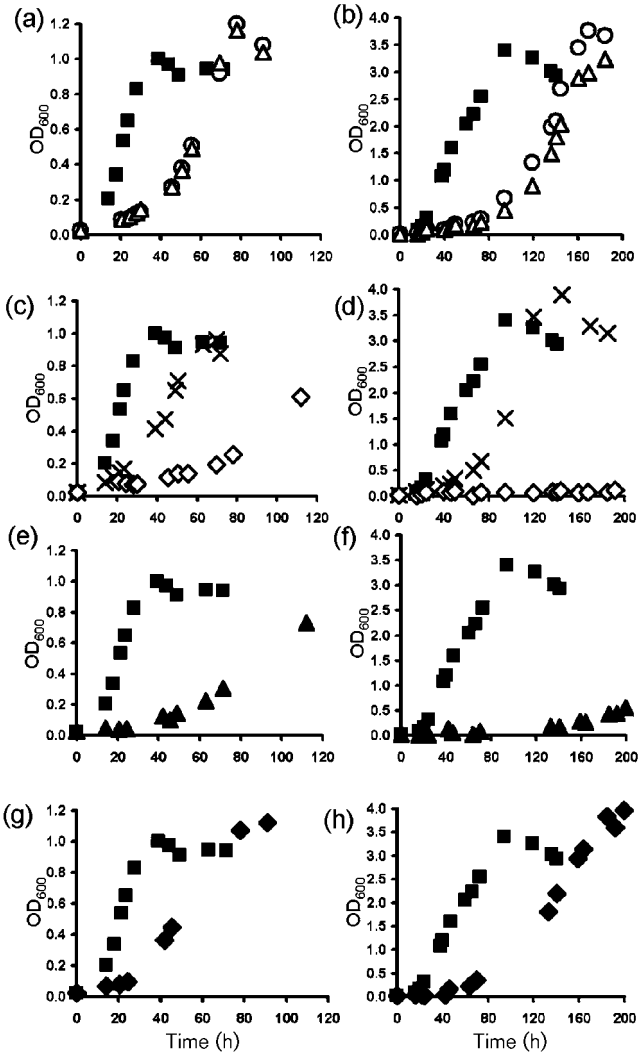

Fig. 2. Propionate metabolism and detoxification via the methylcitrate cycle. (a–h) Bacteria were grown at 37 °C with aeration in M9 broth containing 0.1 % (a, c, e, g) or 0.5 % (b, d, f, h) propionate and culture turbidity (OD_{600}) was measured at the indicated time points. Bacterial strains: ■, wild-type (a–h); ○, Δ*prpDBC* (a, b); △, Δ*prpDBC* Δ*icl1* Δ*icl2* (a, b); ×, Δ*prpB* (c, d); ◇, Δ*prpB* Δ*icl1* Δ*icl2* (c, d); ▲, Δ*prpDBC* Δ*icl1* Δ*icl2* transformed with pPRPDC (e, f); ◆, Δ*prpB* Δ*icl1* Δ*icl2* transformed with pPRPB (g, h). Data from one experiment are shown. Results are representative of at least two experiments with similar results.

0.1 % glucose as the sole carbon source (Table 2). This activity was increased dramatically in extracts from wild-type cells grown in media containing propionate at 0.1 % (>50-fold induction) or 0.5 % (>80-fold induction) (Table 2). Surprisingly, MCL activity was reduced only modestly (by about twofold) in extracts from propionate-grown Δ*prpDBC* bacteria as compared to wild-type bacteria (Table 2). These observations suggest that *M. smegmatis* possesses another gene(s), in addition to *prpB*, that encodes MCL activity.

Table 2. MCS, MCL and ICL enzyme activities in *M. smegmatis* cell-free extracts

Strain	Carbon source	MCS activity*	MCL activity†	ICL activity‡	Ratio ICL/MCL
WT	0.1 % Glucose	Undetectable	2.32 ± 0.31	15.24 ± 2.01	6.57
Δ*prpDBC*		Undetectable	2.52 ± 1.51	11.51 ± 0.14	4.58
Δ*prpDBC* Δ*icl1* Δ*icl2*		ND	Undetectable	Undetectable	–
WT	0.1 % Propionate	ND	124.43 ± 27.15	85.03 ± 6.92	0.68
Δ*prpDBC*		ND	49.65 ± 3.94	254.33 ± 21.97	5.10
Δ*prpDBC* Δ*icl1* Δ*icl2*		ND	Undetectable	Undetectable	–
WT	0.5 % Propionate	11.47 ± 0.94	190.80 ± 0.72	196.78 ± 44.25	1.03
Δ*prpDBC*		Undetectable	76.02 ± 5.71	420.67 ± 26.57	5.50
Δ*prpDBC* Δ*icl1* Δ*icl2*		ND	Undetectable	Undetectable	–

*Units are defined as mean nanomoles of oxaloacetate-stimulated free CoA production from propionyl-CoA min^{-1} (mg protein)$^{-1}$ in the cell-free extract ± SD. Detection limit: 1 nmol min^{-1} mg^{-1}. Three measurements were made per extract. Data from one experiment are shown. Results are representative of at least two experiments with similar results. ND, not done.

†Units are defined as mean nanomoles of 2-methylisocitrate-stimulated NADH consumption min^{-1} (mg protein)$^{-1}$ in the cell-free extract ± SD. Detection limit: 1 nmol min^{-1} mg^{-1}. Three measurements were made per extract. Data from one experiment are shown. Results are representative of at least two experiments with similar results.

‡Units are defined as mean nanomoles of isocitrate-stimulated NADH consumption min^{-1} (mg protein)$^{-1}$ in the cell-free extract ± SD. Detection limit: 1 nmol min^{-1} mg^{-1}. Three measurements were made per extract. Data from one experiment are shown. Results are representative of at least two experiments with similar results.

WT, wild-type.

In *M. tuberculosis*, which lacks a *prpB* homologue, the *icl1* and *icl2* genes encode bifunctional enzymes with ICL and MCL activities (Gould *et al.*, 2006; Munoz-Elias *et al.*, 2006). The *M. smegmatis* ICL1 and ICL2 proteins are highly homologous to their counterparts in *M. tuberculosis* (92 % and 77 % identical, respectively), suggesting that they too might be bifunctional enzymes. To test this idea, we deleted *prpDBC* in wild-type and Δ*icl1* Δ*icl2* strains of *M. smegmatis*, which grow with similar kinetics in propionate-containing media (Ernesto Muñoz-Elías and Lubomir Merkov, The Rockefeller University, New York, personal communication). The Δ*prpDBC* Δ*icl1* Δ*icl2* strain grew normally on media containing glucose as the sole carbon source (not shown); this strain was delayed for growth on media containing propionate at 0.1 % (Fig. 2a) or 0.5 % (Fig. 2b), but only to a similar extent as the parental Δ*prpDBC* strain. We conclude that ICL1/ICL2 are not required for propionate metabolism in the presence or absence of a functional methylcitrate cycle. Nonetheless, the ICL and MCL activities that we detected in extracts from propionate-grown Δ*prpDBC* cells were both, apparently, due to ICL1/ICL2, because both activities were abolished in extracts from Δ*prpDBC* Δ*icl1* Δ*icl2* bacteria (Table 2). Consistent with this interpretation, the ratio of ICL to MCL activities in extracts prepared from Δ*prpDBC* bacteria was similar under all growth conditions that we tested (Table 2). These observations suggest that the *M. smegmatis icl1* and/or *icl2* genes encode bifunctional ICL/MCL enzymes, similar to their orthologues in *M. tuberculosis*.

Given the absence of detectable MCL and MCS activities in the Δ*prpDBC* Δ*icl1* Δ*icl2* cell-free extracts, it is likely that this strain lacks a functional methylcitrate cycle. This conclusion is reinforced by our observation that the presence or absence of *icl1* and *icl2* had little or no effect on the growth kinetics of Δ*prpDBC* bacteria in propionate-containing media, suggesting that there are no alternative sources of MCS or MCD activities other than the *prpC* and *prpD* genes, respectively. In the absence of the methylcitrate cycle, growth of the Δ*prpDBC* and Δ*prpDBC* Δ*icl1* Δ*icl2* strains on propionate might be attributable to the activity of one or more alternative pathways of propionate metabolism that have been proposed (Horswill & Escalante-Semerena, 1999; Textor *et al.*, 1997).

MCL deficiency sensitizes *M. smegmatis* to propionate toxicity

The observation that wild-type and Δ*icl1* Δ*icl2* strains of *M. smegmatis* grow with similar kinetics in propionate-containing media suggests that *prpB* can fully compensate for the loss of ICL1/ICL2 under these conditions. In order to determine whether the converse might also be true, we constructed an unmarked non-polar deletion of *prpB* in which codon 5 was fused in-frame to codon 303 (see Methods), leaving the *prpD* and *prpC* genes intact. As expected, deletion of *prpB* in either the wild-type or Δ*icl1* Δ*icl2* genetic background had no discernible impact on bacterial growth with glucose as the carbon source (not shown). The Δ*prpB* strain was partially impaired for growth on 0.1 % (Fig. 2c) or 0.5 % (Fig. 2d) propionate, but was more robust than Δ*prpDBC* or Δ*prpDBC* Δ*icl1* Δ*icl2* bacteria under these conditions (Fig. 2a, b), consistent with the idea that ICL1/ICL2 are bifunctional ICL/MCL enzymes that can compensate, at least partially, for the loss of *prpB*.

Paradoxically, Δ*prpB* Δ*icl1* Δ*icl2* bacteria (Fig. 2c) were much more severely impaired than Δ*prpDBC* Δ*icl1* Δ*icl2* bacteria (Fig. 2a) for growth on 0.1 % propionate. Moreover, this strain was completely unable to grow in medium containing 0.5 % propionate (Fig. 2d), although there was no loss of viability during the course of the experiment (data not shown). This striking phenotype was reversed by complementation of the Δ*prpB* Δ*icl1* Δ*icl2* strain with a plasmid containing an intact copy of the *prpB* gene, confirming that the phenotype was due to loss of MCL activity (Fig. 2 g, h). These observations suggest that the presence of *prpC* and/or *prpD* might be detrimental to growth on propionate-containing media in the absence of MCL activity. Consistent with this idea, we found that transformation of the *M. smegmatis* Δ*prpDBC* Δ*icl1* Δ*icl2* strain with a plasmid containing the *M. tuberculosis prpDC* genes (Munoz-Elias *et al.*, 2006) resulted in reduced growth on 0.1 % propionate (Fig. 2e) and nearly complete growth inhibition on 0.5 % propionate (Fig. 2f), similar to the phenotype of Δ*prpB* Δ*icl1* Δ*icl2* bacteria (Fig. 2c, d). These observations indicate that in propionate-metabolizing bacteria, loss of MCL activity alone is more detrimental than loss of the entire methylcitrate cycle.

Propionate toxicity is not relieved by the addition of glucose to the growth medium

Our observations that propionate toxicity towards MCL-deficient bacteria is dose-dependent and requires MCS/MCD activity (Fig. 2) suggest that the accumulation of propionate metabolites might adversely affect the expression or activity of propionate-metabolizing pathways other than the methylcitrate cycle. Alternatively, we considered the possibility that propionate metabolites might exert more general growth-inhibitory effects, based on our previous observation that growth of *prpDC*-deficient *M. tuberculosis* on acetate- or glucose-containing media is unimpaired, whilst growth on mixtures of acetate plus propionate or glucose plus propionate is inhibited (Munoz-Elias *et al.*, 2006). We therefore tested whether *M. smegmatis* strains lacking MCL and/or MCS/MCD activities were capable of growth on mixed substrates. Compared to the parental strain, growth of the Δ*prpDBC* (Fig. 3a), Δ*prpDBC* Δ*icl1* Δ*icl2* (Fig. 3a) and Δ*prpB* (Fig. 3b) strains was delayed and growth of the Δ*prpB* Δ*icl1* Δ*icl2* strain (Fig. 3b) was almost completely inhibited in medium containing a mixture of glucose plus propionate. Complementation of the Δ*prpB* Δ*icl1* Δ*icl2* strain with a plasmid encoding *prpB* restored growth on mixed-substrate media (glucose plus propionate) to nearly wild-type levels (Fig. 3d). As observed when the bacteria were grown on propionate as the sole carbon source (Fig. 2e, f), restoration of the *prpDC* genes to Δ*prpDBC* Δ*icl1* Δ*icl2* bacteria paradoxically impaired rather than enhanced growth on glucose plus propionate (Fig. 3c). These observations reinforce the idea that MCS/MCD-generated propionate metabolites exert a dominant inhibitory effect on bacterial growth that is especially pronounced in the absence of MCL activity.

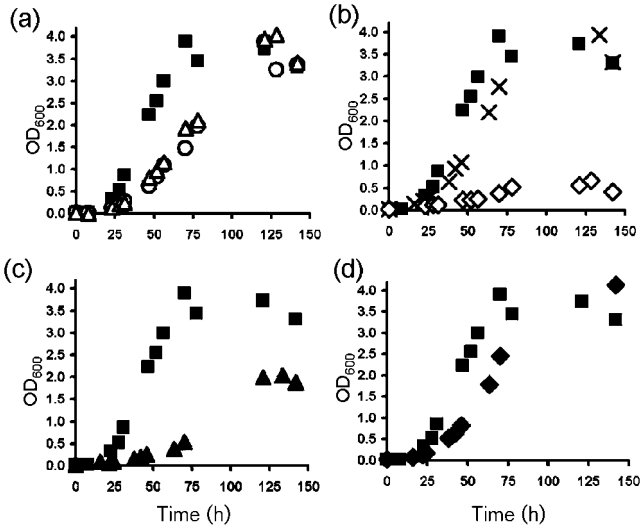

Fig. 3. Propionate toxicity is substrate-dominant and potentiated by MCL deficiency. (a–d) Bacteria were grown at 37 °C with aeration in M9 broth containing 0.1 % glucose and 0.5 % propionate and culture turbidity (OD_{600}) was measured at the indicated time points. Bacterial strains: ■, wild-type (a–d); ○, Δ*prpDBC* (a); △, Δ*prpDBC* Δ*icl1* Δ*icl2* (a); ×, Δ*prpB* (b); ◇, Δ*prpB* Δ*icl1* Δ*icl2* (b); ▲, Δ*prpDBC* Δ*icl1* Δ*icl2* transformed with pPRPDC (c); ◆, Δ*prpB* Δ*icl1* Δ*icl2* transformed with pPRPB (d). Data from one experiment are shown. Results are representative of at least two experiments with similar results.

DISCUSSION

Propionyl-CoA is derived from the catabolism of propionate, branched-chain amino acids and odd-chain-length fatty acids, which are abundant carbon/energy sources for soil-dwelling micro-organisms and commensals of the mammalian intestinal tract (Chauhan & Ogram, 2006; Conrad & Klose, 1999, 2000; Munoz-Elias & McKinney, 2006), but there have been few studies on the role of propionyl-CoA metabolism in pathogenic microbes. Several pathways of microbial propionate metabolism have been proposed (Horswill & Escalante-Semerena, 1999; Textor *et al.*, 1997). Genes encoding the corresponding enzymes are found in diverse species, including some pathogenic bacteria and fungi (Horswill & Escalante-Semerena, 1999; Maerker *et al.*, 2005; Munoz-Elias *et al.*, 2006; Stone *et al.*, 1999), suggesting that propionyl-CoA might be an important carbon/energy source during infection. However, propionate also possesses antifungal and antibacterial properties and propionate strongly inhibits growth of some micro-organisms, even in the presence of other carbon sources (Brock *et al.*, 2000; Claes *et al.*, 2002). In species that are well adapted to environments containing high levels of propionate, it is unclear whether the principal role of propionate metabolism is in carbon/energy metabolism or detoxification.

The methylcitrate cycle (Fig. 1a) is a major pathway of propionate metabolism that is widely distributed among bacteria and fungi (reviewed by Munoz-Elias & McKinney, 2006; Munoz-Elias *et al.*, 2006). We reported previously that *M. tuberculosis* requires this pathway for propionate metabolism *in vitro*, despite encoding only two of three enzymes specific to the methylcitrate cycle, MCS (*prpC*) and MCD (*prpD*) (Munoz-Elias *et al.*, 2006). *M. tuberculosis* lacks the final enzyme, MCL (*prpB*), and this activity is instead provided by two bifunctional ICL/MCL enzymes encoded by the *icl1* and *icl2* genes (Munoz-Elias *et al.*, 2006). The bifunctional ICL1/ICL2 participate in both the glyoxylate cycle as ICL (Fig. 1b) and the methylcitrate cycle as MCL (Fig. 1a) and are therefore required for growth *in vitro* on either acetate or propionate, respectively (Gould *et al.*, 2006; Munoz-Elias & McKinney, 2005; Munoz-Elias *et al.*, 2006). At least one of these pathways appears to be essential for bacterial metabolism during infection because ICL1/ICL2 are jointly required for survival of *M. tuberculosis* in macrophages and mice (Munoz-Elias & McKinney, 2005). Selective disruption of the glyoxylate cycle could be accomplished by deletion of the *glcB* gene encoding MLS, which is not involved in the methylcitrate cycle. Selective disruption of the methylcitrate cycle by deletion of the *prpDC* locus paradoxically attenuates *M. tuberculosis* replication in macrophages but not in mice (Munoz-Elias *et al.*, 2006), suggesting that loss of the methylcitrate cycle might be buffered by induction of another pathway for propionate metabolism when the bacteria are grown *in vivo*.

Several lines of evidence suggest that fatty acids might serve as an important source of carbon and energy for *M. tuberculosis* during infection (reviewed by Boshoff & Barry, 2005; Munoz-Elias & McKinney, 2006), which could explain why ICL1/ICL2 are required for *in vivo* survival. However, recent studies on the mechanism of propionate toxicity in other micro-organisms (Brock & Buckel, 2004; Brock, 2005; Horswill *et al.*, 2001) suggested the possibility that ICL1/ICL2 might also serve a critical role in the removal of potentially toxic propionate metabolites *in vivo*. Here, using *M. smegmatis*, a fast-growing relative of *M. tuberculosis*, we provide evidence that ICL/MCL activity is indeed required for removal of toxic methylcitrate cycle metabolites during growth on propionate-containing media, even in the presence of other carbon sources (such as glucose) that are metabolized by non-overlapping pathways. Unlike *M. tuberculosis* (Munoz-Elias *et al.*, 2006), *M. smegmatis* apparently uses but does not absolutely require the methylcitrate cycle for growth on propionate-containing media, indicating the existence of an alternative pathway(s) for propionate metabolism in this species.

Among microbes that metabolize propionate via the methylcitrate cycle, *M. smegmatis* is so far unique in producing a monofunctional MCL (*prpB*) as well as bifunctional ICL/MCL (*icl1*, *icl2*). In other well-characterized species that operate both the glyoxylate and methylcitrate cycles, ICL and MCL activities are encoded by distinct genes with non-overlapping functions (Bramer & Steinbuchel, 2001; Brock *et al.*, 2001; Brock, 2005; Claes *et al.*, 2002; Horswill & Escalante-Semerena, 1999). The *M. smegmatis prpB* gene product contains the active site catalytic motif KRCGH, which is characteristic of MCL (Brock *et al.*, 2001), and this enzyme apparently cannot substitute for ICL1/ICL2 in the glyoxylate cycle (Ernesto Muñoz-Elías and Lubomir Merkov, personal communication). In contrast, although the *M. smegmatis icl1* and *icl2* gene products contain the KK/QCGH motif previously associated only with monofunctional ICL (Brock *et al.*, 2001), they appear to participate in both the glyoxylate cycle and the methylcitrate cycle, like their orthologues in *M. tuberculosis* (Munoz-Elias *et al.*, 2006). The participation of mycobacterial ICL1/ICL2 in both pathways might reflect a unique ability of these enzymes to cleave isocitrate as well as methylisocitrate (Gould *et al.*, 2006), or might instead reflect differential regulation of ICL expression in mycobacteria as compared to other species.

Also in contrast to some other microbes, which require the glyoxylate cycle (Fig. 1b) for carbon anaplerosis during propionate metabolism (Ashworth & Kornberg, 1964; Bramer & Steinbuchel, 2001; Wang *et al.*, 2003; Wegener *et al.*, 1969), we found that growth of *M. smegmatis* on propionate as the sole carbon source was not affected by deletion of *icl1* and *icl2*, in either the presence or absence of the methylcitrate cycle (see below), nor by deletion of the *glcB* gene encoding MLS (unpublished observations). Anaplerotic enzymes that might substitute for the glyoxylate cycle under these conditions include malic enzyme (*mez; msm5055*), pyruvate carboxylase (*pca; msm2412, msm6648*), or PEP synthase (*msm3934*) and PEP carboxylase (*msm3097*). Although ICL1/ICL2 were not required for growth of *M. smegmatis* on propionate as the sole carbon source, the upregulation of their activities under these conditions suggests that the glyoxylate cycle might be a preferred (but not essential) anaplerotic route in cells metabolizing propionate. The highest levels of ICL activity were observed in extracts from Δ*prpDBC* bacteria, suggesting the possibility of regulatory crosstalk between the *icl* and *prp* loci.

Unexpectedly, we found that deletion of the *prpDBC* locus reduced, but did not eliminate, growth of *M. smegmatis* on propionate as the sole carbon source. In contrast, *M. tuberculosis* absolutely requires the *prpDC* locus for growth on propionate-containing media (Munoz-Elias *et al.*, 2006). These observations suggest that *M. smegmatis* possesses an alternative route(s) of propionate metabolism that is absent or less efficient in *M. tuberculosis*. Potential routes of propionate metabolism include the methylmalonyl-CoA pathway, which dominates propionyl-CoA metabolism in mammalian cells. The *M. smegmatis* genome contains orthologues of all of the genes specific to the methylmalonyl-CoA pathway, as does the *M. tuberculosis* genome (Cole *et al.*, 1998; Fleischmann *et al.*, 2002), and propionyl-CoA carboxylase, catalysing the first dedicated reaction in this pathway, has been demonstrated in cell-free

extracts from both species (Wheeler *et al.*, 1992). Further work is required to determine whether the methylmalonyl-CoA pathway is responsible for growth of the *M. smegmatis* Δ*prpDBC* strain on propionate-containing media and, if so, why this pathway apparently cannot support growth of the *M. tuberculosis* Δ*prpDC* strain under these conditions. The delayed growth of Δ*prpDBC M. smegmatis* after transfer to propionate-containing media might reflect the time required for induction of alternative metabolic pathways. Delayed growth was observed when Δ*prpDBC* bacteria were transferred to media containing propionate alone or a mixture of glucose plus propionate. In the latter case, glucose metabolism might be blocked by the accumulation of propionyl-CoA, which has been shown to inhibit pyruvate dehydrogenase in *Aspergillus* spp. (Brock & Buckel, 2004; Maerker *et al.*, 2005) and *Rhodobacter sphaeroides* (Maruyama & Kitamura, 1985).

Paradoxically, we found that the residual growth of Δ*prpDBC* Δ*icl1* Δ*icl2* bacteria in media containing propionate, or a mixture of propionate plus glucose, was abolished by restoration of the *prpDC* genes. Growth inhibition of this strain by propionate was also dose-dependent, suggesting that the propionate metabolites generated by MCS/MCD are toxic in the absence of MCL activity. Substrate-dominant growth inhibition by propionate has also been observed in methylcitrate cycle mutants of *A. nidulans* (Brock, 2005) and *S. typhimurium* (Horswill *et al.*, 2001), which accumulate MCS/MCD-generated propionate metabolites. Although the mechanism of growth inhibition by propionate metabolites is unknown, proposed molecular targets include isocitrate dehydrogenase, aconitase and citrate synthase (Brock, 2005; Cheema-Dhadli *et al.*, 1975).

Our discovery that MCL activity is critical for detoxification of MCS/MCD-generated propionate metabolites in *M. smegmatis* calls for a re-evaluation of our earlier studies on the metabolism of *M. tuberculosis* during infection. Previously we interpreted our observation that ICL1/ICL2 are jointly required for survival of *M. tuberculosis* in mice as indicative of a central role for fatty acid catabolism during infection (Munoz-Elias & McKinney, 2005). However, the new findings reported here suggest an alternative explanation, that ICL1/ICL2 might be required *in vivo* for the detoxification of MCS/MCD-generated propionate metabolites. Consistent with this interpretation, a recent analysis of cell wall-associated lipids in *M. tuberculosis* recovered from mouse lungs suggests that the bacteria accumulate high levels of propionyl-CoA during growth in this environment (Jain *et al.*, 2007). This conclusion is also suggested by the marked increase in expression of the *prpDC* locus when *M. tuberculosis* is grown in macrophages or in the lungs of mice (Mattow *et al.*, 2006; Schnappinger *et al.*, 2003). These observations suggest that deletion of ICL1/ICL2 might result in growth inhibition due to accumulation of toxic MCS/MCD-generated propionate metabolites. If this hypothesis is correct, then deletion of *prpDC* should paradoxically relieve, in whole or in part, the *in vivo* requirement for ICL1/ICL2. In this context it is noteworthy that deletion of *prpD* alone might attenuate the growth of *M. tuberculosis* in mice (unpublished preliminary data cited in Mattow *et al.*, 2006), whereas deletion of both *prpC* and *prpD* did not (Munoz-Elias *et al.*, 2006), suggesting that MCS-generated propionate metabolites might be growth inhibitory in the absence of MCD activity. In light of the new findings reported here, further studies are needed to clarify whether the essential function of *M. tuberculosis* ICL1/ICL2 during infection is attributable to their role in the glyoxylate cycle (fatty acid catabolism), the methylcitrate cycle (propionyl-CoA metabolism/detoxification), or both pathways.

ACKNOWLEDGEMENTS

The authors thank Ernesto Muñoz-Elías and Lubomir Merkov for generously providing the *M. smegmatis* Δ*icl1* Δ*icl2* strain and for communicating their unpublished results. This work was supported by National Institutes of Health Grant HL088906 (to J. D. M.).

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Edited by: G. S. Besra