Functional Characterization of a Vitamin B$_{12}$-Dependent Methylmalonyl Pathway in *Mycobacterium tuberculosis*: Implications for Propionate Metabolism during Growth on Fatty Acids$^{V}$†

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*Mycobacterium tuberculosis* is predicted to subsist on alternative carbon sources during persistence within the human host. Catabolism of odd- and branched-chain fatty acids, branched-chain amino acids, and cholesterol generates propionyl-coenzyme A (CoA) as a terminal, three-carbon (C$_3$) product. Propionate constitutes a key precursor in lipid biosynthesis but is toxic if accumulated, potentially imparting its metabolism in *M. tuberculosis* pathogenesis. In addition to the well-characterized methylcitrate cycle, the *M. tuberculosis* genome contains a complete methylmalonate pathway, including a mutAB-encoded methylmalonyl-CoA mutase (MCM) that requires a vitamin B$_{12}$-derived cofactor for activity. Here, we demonstrate the ability of *M. tuberculosis* to utilize propionate as the sole carbon source in the absence of a functional methylcitrate cycle, provided that vitamin B$_{12}$ is supplied exogenously. We show that this ability is dependent on mutAB and, furthermore, that an active methylmalonate pathway allows the bypass of the glyoxylate cycle during growth on propionate in vitro. Importantly, although the glyoxylate and methylcitrate cycles supported robust growth of *M. tuberculosis* on the C$_{17}$ fatty acid heptadecanoate, growth on valerate (C$_5$) was significantly enhanced through vitamin B$_{12}$ supplementation. Moreover, both wild-type and methylcitrate cycle mutant strains grew on B$_{12}$-supplemented valerate in the presence of 3-nitropropionate, an inhibitor of the glyoxylate cycle enzyme isocitrate lyase, indicating an anaplerotic role for the methylmalonaldehyde pathway. The demonstrated functionality of MCM reinforces the potential relevance of vitamin B$_{12}$ to mycobacterial pathogenesis and suggests that vitamin B$_{12}$ availability in vivo might resolve the paradoxical dispensability of the methylcitrate cycle for the growth and persistence of *M. tuberculosis* in mice.

*Mycobacterium tuberculosis* is an obligate human pathogen that is expected to adapt metabolically to conditions that are often hostile and nutrient poor during successive cycles of infection, replication, persistence, and transmission. In particular, glucose deficiency and an abundance of fatty acids are thought to dictate mycobacterial metabolism during infection (3, 35), consistent with the complex repertoire of genes involved in lipid metabolism in the *M. tuberculosis* genome (10). Subsistence on fatty acids requires the sequential action of the catabolic β-oxidation cycle and, where glycolytic substrates are limiting, the anaplerotic glyoxylate cycle, which enables the assimilation of derivative two-carbon (C$_2$) acetyl-coenzyme A (CoA) subunits (37). In addition to producing acetyl-CoA, β-oxidation of odd- and branched-chain fatty acids yields the C$_3$ subunit propionyl-CoA. This metabolite can also be generated by the catabolism of branched-chain amino acids (24) and cholesterol. Recently, a cassette of genes involved in the catabolism of the A and B rings of cholesterol to propionyl-CoA, pyruvate, and other metabolites was identified in actinomyces, including members of the *M. tuberculosis* complex (27, 52). Although the relevance of cholesterol as a carbon source for *M. tuberculosis* in vivo has yet to be established, the likely action of this catabolic pathway during intracellular growth and survival of *M. tuberculosis* (52) suggests that it may constitute an additional, and potentially significant, source of propionyl-CoA in this pathogen.

Propionyl-CoA is a key precursor in several lipid biosynthetic pathways in *M. tuberculosis* (28); however, while providing a high-energy metabolite, the accumulation of propionate is toxic to the cell, and as such, efficient mechanisms are required for its disposal (5). This dual nature implies a central role for propionate metabolism in the growth and persistence of *M. tuberculosis* in vivo (18, 37). Evidence that a shift to catabolism of host lipids potentiates *M. tuberculosis* virulence through the increased biosynthesis of the virulence factors phthiocerol dimycocerosate and sulfolipid 1 (25) strengthens this contention. Recently, the possibility that the methylcitrate cycle might constitute the dominant pathway for propionate metabolism in vivo was investigated (37). The two key findings that motivated this investigation were the observed upregulation of methylcitrate cycle genes in the intracellular environment and in the mouse lung (34, 48) and the inability of a ΔiclII
Δisc2 double mutant of *M. tuberculosis* Erdman to grow on propionate in vitro or establish an infection in mice (36). The unusual involvement of *icll* and *icl2* in both the methylcitrate cycle (as 2-methylisocitrate lyase [MCL]) and the glyoxylate cycle (as isocitrate lyase [ICL]) (18, 37) in *M. tuberculosis*, however, complicates any interpretation of the relative importance of these pathways to *M. tuberculosis* metabolism. Moreover, the demonstration by Muñoz-Elías et al. that a mutant of *M. tuberculosis* Erdman lacking two earlier genes in the methylcitrate pathway, *pppD*, encoding methylcitrate dehydratase (MCD), and *pppC*, encoding methylcitrate synthase (MCS), is unable to grow on propionate in vitro but establishes a wild-type infection in mice suggested the possibility that propionate might be oxidized via an alternative route in vivo (37).

The methylmalonyl pathway offers a potentially attractive alternative to the methylcitrate cycle (8, 38, 49, 51, 54, 55); however, the function of this pathway and its role in propionate metabolism in *M. tuberculosis* has remained unexplored. The final step in the methylmalonyl pathway is the reversible intramolecular rearrangement of (R)-methylmalonyl-CoA to succinyl-CoA (Fig. 1). This reaction is catalyzed by the *mutAB*-encoded methylmalonyl-CoA mutase (MCM), a vitamin B12-dependent enzyme (33). We sought to address whether the *mutAB*-encoded MCM is functional in *M. tuberculosis* and to investigate the possibility that the methylmalonyl pathway provides an alternative to the methylcitrate cycle during growth on propionate. During concurrent studies on other vitamin B12-dependent enzymes in *M. tuberculosis* (56), we demonstrated the functionality of the B12-dependent methionine synthase (MetH) and the operation of a B12-dependent regulatory mechanism (a B12 riboswitch) (56), potentially implicating vitamin B12 metabolism in *M. tuberculosis* pathogenesis. Importantly, those studies revealed that *M. tuberculosis* does not produce vitamin B12 in vitro but has the capacity to transport and utilize this cofactor when exogenously supplied in the form of cyanocobalamin. Although the extent to which vitamin B12 availability dictates the activity of the B12-dependent enzymes in vivo remains unclear, the implication of these observations for the function of the vitamin B12-dependent MCM was immediately evident. In this paper, we demonstrate the functionality of the methylmalonyl pathway in *M. tuberculosis* under conditions in which vitamin B12 is not limiting and discuss the implications of these findings for the growth of *M. tuberculosis* on fatty acid substrates.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used in this study are detailed in Table S1 in the supplemental material. Wild-type *M. tuberculosis* strain H37Rv (ATCC 25618) and mutant derivatives thereof were grown standing at 37°C in Middlebrook 7H9 medium (Merck) supplemented with 0.2% glycerol, oleic acid-albumin-dextrose-catalase enrichment (Merck), and 0.05% Tween 80. Sodium propionate, valeric acid, and heptadecanoic acid were purchased from Sigma. For carbon utilization experiments, bacteria were grown in 7H9 medium containing 0.5% albumin, 0.0085% NaCl, 0.05% Tween 80, and sodium propionate or valeric acid at a concentration of 0.1% (10 mM in both cases). The pH of the valeric acid-containing medium was adjusted to 6.8 with 10 M NaOH prior to use. In the case of heptadecanoic acid, a prewarmed 0.2% stock solution of heptadecanoic acid was added to the medium at a final concentration of 0.007% (0.25 mM). The lower final concentration of the carbon source in this case was attributable to the poor solubility of heptadecanoic acid in water, which limited the concentration that could be achieved. Unless otherwise indicated, vitamin B12, supplement (cyanocobalamin; Sigma) was included at a concentration of 10 μg/mL. Hygromycin and kanamycin were used in *M. tuberculosis* cultures at final concentrations of 50 and 25 μg/mL, respectively, and where indicated, 3-nitropropionate (3NP) (Sigma) was used at a concentration of 0.1 mM (36).

**Construction of mutant strains.** A 7,660-bp EcoRI fragment of *M. tuberculosis* genomic DNA carrying the *mutAB* genes was obtained from the H37Rv bacterial artificial chromosome library clone Rv58 (7) and cloned into p2mutE (43) to form p2mutAB. An internal, 2,342-bp region of *mutAB* was deleted from p2mutAB by digestion with AscI and BglII. The fragment was blunt ended with Klencow fragment (Roche) and religated to create p2mutAB. The *mutAB* mutation created an out-of-frame fusion at the AscI/BglII junction and eliminated 213 amino acids from the C terminus of the 615-amino-acid MutA and 566 amino acids from the N terminus of the 750-amino-acid MutB (see Fig. S1 in the supplemental material). The lacZ-sacB marker gene cassette from pG0AL17 (43) was then inserted into the Pael site of p2mutAB, which was used to construct the *mutAB* mutant of *M. tuberculosis* H37Rv by standard two-step allelic exchange mutagenesis using previously described methods (17, 43). Genetic reversion of the *mutAB* mutation in the *mutAB* mutant strain was carried out by knock-in allelic exchange mutagenesis using the suicide plasmid p2mutAB17, which was constructed to introduce the *mutAB* genes in the *mutAB* mutant strain by homologous recombination using the suicide plasmid p2mutAB17, which contains the full-length *mutAB* genes plus 1,431 bp of 3′- and 2,228 bp of 5′-flanking chromosomal sequences (see Table S1 in the supplemental material) and was produced by cloning the lacZ-sacB cassette from pG0AL17 into the Pael site of p2mutAB. The *ΔψPrpDC*, *ΔmutAB*, *ΔψPrpDC*, and *ΔmutAB*/*ΔmutAB* ΔψPrpDC mutants were constructed by the deletion of *ψPrpDC* in the H37Rv, *ΔmutAB*, and *ΔmutAB*/*mutAB* backgrounds, respectively, using the previously described suicide plasmid pAU100 (37). The *ΔψPrpDC* mutant was complemented genetically by the integration of pPRPDC at the attB locus (37). All mutant strains were genotypically confirmed by Southern blot analysis, as previously described (17; data not shown) (see Fig. S1 in the supplemental material).

**Gene expression analysis by real-time qRT-PCR.** The level of expression of the *prpD* gene (Rv1130) in H37Rv cells cultured under various conditions was determined by real-time quantitative reverse transcription-qPCR (qRT-PCR). Cultures were grown to mid-log phase (optical density at 600 nm [OD600] of 0.4), bacteria were harvested, and RNA was extracted using TRIzol (Sigma). RNA (0.5 to 2.5 μg) was used to synthesize cDNA using previously described methods (13, 26). Real-time qRT-PCR was carried out using 2 μl of cDNA for amplification with the LightCycler FastStart DNA Master Sybr green I kit with Roche LightCycler software (version 1.5), and absolute quantifications of transcript levels using standard curves were performed with LightCycler software (version 4.0) (26). The primers used to determine *prpD* transcript levels were PRPDF (5′-GGTCTGGATAACCCGCTATGA) and PRPRD (5′-ATCGCGTGGTAGAATGCCTGTA) TGTCCTC), and those used to determine *ψPrpDC* transcript levels for normalization were described previously by Dawes et al. (12). The paired t test was used to assess the statistical significance of pairwise comparisons using GraphPad Prism software (http://www.graphpad.com/quickcalcs/ttest1.cfm).

**RESULTS**

**Identification of genes encoding the methylmalonyl pathway.** The key elements of propionate metabolism deduced from the genome sequence of *M. tuberculosis* H37Rv and determined experimentally (18, 37) are illustrated in Fig. 1. The methylcitrate cycle converts propionyl-CoA and oxaloacetate to pyruvate and succinate and was described previously (37). It comprises MCS and MCD enzymes encoded by *pppC* and *pppD*, respectively. *M. tuberculosis* is unusual in not encoding a dedicated MCL and, instead, relies on the glyoxylate cycle enzyme Icl1 and Icl2, in strains of *M. tuberculosis* possessing a functional version of this enzyme) for both ICL and MCL activity (18, 37).

The methylmalonyl pathway, on the other hand, converts propionyl-CoA to succinyl-CoA via a methylmalonyl-CoA intermediate. In the first step, propionyl-CoA carboxylase (PCC) synthesizes (3)-methylmalonyl-CoA from propionyl-CoA. The PCC complex in *M. tuberculosis*, which has been characterized biochemically, comprises α, β, and ε subunits encoded by...
accA3 (Rv3285), accD5 (Rv3280), and accE5 (Rv3281), respectively (14, 32); notably, both accA3 and accE5 are predicted to be essential for the optimal growth of M. tuberculosis in vitro (47). Methylmalonyl-CoA epimerase (MMCE) then catalyzes the conversion of (S)-methylmalonyl-CoA to (R)-methylmalonyl-CoA, the epimer necessary for subsequent B12-dependent MCM activity. Based on a BLAST analysis (1), we have assigned Rv1322A as the M. tuberculosis MMCE with approximately 40% identity and 60% similarity to characterized MMCEs from other bacteria (2, 30). The final reaction of the methylmalonyl pathway, the isomerization of (R)-methylmalonyl-CoA to succinyl-CoA, is catalyzed by MCM, a heterodimer comprising subunits encoded by mutA (Rv1492) and mutB (Rv1493) (33). The α-subunit, MutB, contains the binding domain for the vitamin B12-derived cofactor adenosylcobalamin. A GTPase, MeaB, functions in the assembly and protection of MCM in other bacteria (22, 29, 40); a BLAST homology search (1) identified Rv1496 as being the putative M. tuberculosis meaB ortholog (57% identity and 70% similarity to MeaB from other organisms). Consistent with this designation,
Rv1496 is located only 626 bp downstream of mutB, with the two genes separated by a predicted MazEF-type toxin-antitoxin module (Rv1494 and Rv1495) (42, 57).

Vitamin B12 supplementation enables growth of a prpDC mutant of M. tuberculosis H37Rv on propionate.

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As shown in Fig. 2A, the prpDC mutant of H37Rv was unable to grow in liquid medium containing propionate as the sole carbon source, recapitulating precisely the phenotype of the corresponding prpDC mutant of Erdman (37).

The phenotype of the prpDC mutant strongly implied the essentiality of the methylcitrate cycle for propionate metabolism in M. tuberculosis (37). Recently, we showed that supplementation of the growth medium with vitamin B12 allowed M. tuberculosis to overcome the loss of the apparently essential (B12-independent) methionine synthase MetE by enabling the activity of the alternative, B12-dependent, methionine synthase MetH (56). To establish that vitamin B12 limitation in vitro was similarly crippling the prpDC-encoded MCM (and, hence, the last step in the methylmalonyl pathway), we supplemented the propionate-containing growth medium with vitamin B12 (Fig. 2B).

The addition of vitamin B12 (as cyanocobalamin) at a
The vitamin $B_12$-dependent growth of the $\Delta prpDC$ mutant on propionate requires $mutAB$. To confirm that the methylmalonyl pathway alone was responsible for both the vitamin $B_12$-dependent growth of the $\Delta prpDC$ mutant on propionate and the vitamin $B_12$-enhanced growth displayed by the wild-type strain, we disrupted MCM function in H37Rv through deletion mutagenesis of $mutAB$ to create the $\Delta mutAB$ mutant. As expected, despite supplementation with vitamin $B_12$, this strain displayed growth kinetics similar to those of H37Rv grown in propionate lacking vitamin $B_12$ (Fig. 2D), suggesting that the enhanced growth seen in $B_12$-supplemented propionate was due solely to the operation of the methylmalonyl pathway. In contrast, the abrogation of both methylcitrate cycle and methylmalonyl pathway function in the $\Delta mutAB$ $\Delta prpDC$ mutant eliminated the ability of $M. tuberculosis$ to metabolize propionate, even in the presence of exogenous vitamin $B_12$ (Fig 2C). Reversion of the $\Delta mutAB$ mutation to wild-type $mutAB$ prior to the introduction of the $\Delta prpDC$ mutation yielded a strain that was able to grow on propionate supplemented with vitamin $B_12$ (Fig. 2C), thus confirming that vitamin $B_12$-dependent growth of $\Delta prpDC$ on propionate is mediated by the $mutAB$-encoded MCM. However, the reversion mutant ($\Delta mutAB::mutAB \Delta prpDC$) did not grow as well as the $\Delta prpDC$ comparator strain (Fig. 2C). The reasons for this difference are unclear, but one possibility is that during the three rounds of allelic exchange mutagenesis required for its construction, the reversion mutant may have inadvertently acquired a second-site mutation(s) that adversely affected its growth on propionate. This difference notwithstanding, these observations nonetheless provided strong evidence for methylmalonyl pathway function in $M. tuberculosis$ and suggested that this pathway provides an alternative to the methylcitrate cycle for growth on propionate where vitamin $B_12$ is not limiting. This notion is consistent with the stimulatory effect of vitamin $B_12$ on the growth of the wild-type strain (Fig. 2D).

A functional methylmalonyl pathway can bypass the requirement for the glyoxylate cycle during growth on propionate. As mentioned above, ICL and MCL activities are encoded by the same gene(s) in $M. tuberculosis$, inextricably linking the functions of the glyoxylate and methylcitrate cycles (37). Furthermore, both glyoxylate and methylcitrate cycles utilize enzymes of the tricarboxylic acid (TCA) cycle, including succinate dehydrogenase, fumarase, and aconitase (Fig. 1). The methylmalonyl pathway, in contrast, is reliant on an autonomous set of enzymes, PCC, MMCE, and MCM, to generate the TCA cycle intermediate succinyl-CoA. This raised the possibility that the methylmalonyl pathway might offer a more efficient route for propionate metabolism, perhaps bypassing the need for anaplerosis via the glyoxylate cycle.

The vitamin $B_12$-dependent growth of the $\Delta prpDC$ mutant on propionate established the ability of the methylmalonyl pathway to support growth in the absence of a functional methylcitrate cycle. However, the sufficiency of the methylmalonyl pathway in the absence of both methylcitrate and glyoxylate cycles remained to be determined. To investigate this, we assayed the growth of H37Rv on propionate while inhibiting Icl1 enzymatic function (ICL and MCL activity) through the addition of 3NP (20). As reported previously for strain Erdman (36), H37Rv was unable to metabolize propionate in the presence of 3NP (Fig. 3A), confirming the essentiality of Icl1 (and Icl2) for the growth of $M. tuberculosis$ on propionate as the sole carbon source under the conditions tested. However, the addition of vitamin $B_12$ appeared to alleviate the 3NP-mediated growth inhibition of the wild-type strain, as evidenced by the growth that eventually occurred, albeit after a prolonged (ca. 2-week) delay (Fig. 3A). Abrogation of MCM activity in the $\Delta mutAB$ mutant eliminated growth on propionate in the presence of 3NP (Fig. 3B). Together, these observations suggested that the methylmalonyl pathway alone is sufficient for the growth of $M. tuberculosis$ on propionate as the sole carbon source, provided that the vitamin $B_12$ cofactor requirement for MCM activity is met.

In contrast to the wild type, no growth delay was observed in the case of the $\Delta prpDC$ mutant, which grew equally well in vitamin $B_12$-supplemented propionate in both the presence and absence of 3NP (Fig. 3A). Since the $\Delta prpDC$ mutation precluded the flux of propionyl-CoA through the methylcitrate cycle, the differential response of wild-type and $\Delta prpDC$ mutant strains to the 3NP-mediated inhibition of both methylcitrate and glyoxylate cycles implicated a buildup of the toxic propionate metabolites 2-methylcitrate and 2-methylisocitrate in the delayed growth of the wild-type strain (21, 44, 50). To investigate whether the growth eventually observed was of wild-type $M. tuberculosis$ or an escape mutant refractory to the inhibitory effects of a methylcitrate cycle intermediate(s), bacteria from the outgrown culture were passaged several times in Middlebrook 7H9 broth to eliminate residual traces of vitamin $B_12$ and then used to inoculate $B_12$-supplemented propionate with or without 3NP. Growth rates in both cases were found to be similar and remained strictly vitamin $B_12$ dependent (data not shown). These observations suggested that a functional methylmalonyl pathway allowed an escape mutant(s) to arise under the pressure imposed by toxic propionate metabolites that accumulated as a result of 3NP-mediated inhibition of the methylcitrate cycle at the step catalyzed by Icl1.

Role of the methylmalonyl pathway in growth of $M. tuberculosis$ on longer odd-chain fatty acids. The data presented above (Fig. 3A and B) established the ability of the methylmalonyl pathway to metabolize propionate independently of both methylcitrate and glyoxylate cycles. However, growth on...
longer odd-chain fatty acids might require the dual operation of both the methylmalonyl pathway and the glyoxylate cycle; that is, partitioning the flux of derivative propionyl-CoA and acetyl-CoA subunits through the methylmalonyl pathway and glyoxylate cycle, respectively, could enable the optimal use of such carbon sources.

To test this possibility, we first assessed the growth of H37Rv on valerate, a C$_5$ substrate which yields acetyl-CoA (C$_2$) and propionyl-CoA (C$_3$) subunits in equal proportion. In agreement with recent evidence (9), H37Rv grew poorly on valerate as the sole carbon source (Fig. 4A). However, supplementation of the medium with vitamin B$_{12}$ improved the growth of the wild-type strain, strongly implying a role for the methylmalonyl pathway in metabolizing the propionyl-CoA derived from this substrate. As observed on propionate-containing medium (Fig. 2A), the prpDC mutant of H37Rv was unable to utilize valerate in the absence of vitamin B$_{12}$ (Fig. 4A) but could grow in vitamin B$_{12}$-supplemented medium, again implying propionate toxicity when both methylcitrate cycle and methylmalonyl pathway functions are crippled. Together, these findings strongly suggested the ability of the methylmalonyl pathway to operate as the preferred route for propionate metabolism under the conditions tested.

Growth of H37Rv and the prpDC mutant was then assessed on the longer odd-chain fatty acid heptadecanoate (C$_{17}$), which produces a much higher ratio of acetyl-CoA to propionyl-CoA subunits (7:1). In contrast to the findings with valerate (Fig. 4A), the wild-type strain displayed robust growth on heptade-
methylmalonyl pathway under conditions in which the prevailing vitamin B₁₂ levels are able to satisfy the cofactor requirements of MCM.

**Differential transcripational response of prpD to propionate versus valerate.** The delayed growth of H37Rv observed on B₁₂-supplemented propionate but not valerate in the presence of 3NP (Fig. 3A versus 5A) suggested that the partitioning of propionyl-CoA between the methylcitrate cycle and the methylmalonyl pathway may differ depending on the carbon source used. To investigate this possibility, we analyzed the expression levels of prpD, normalized to sigA, in various carbon sources (Fig. 6). As observed for other organisms (4, 6, 23, 31, 41), prpD expression in *M. tuberculosis* was markedly induced in propionate compared to that in a propionate-free control (7H9) medium. In vitamin B₁₂-supplemented propionate, the expression level of prpD remained significantly higher than that of the 7H9 control but was halved in comparison with that of propionate without B₁₂ supplementation (Fig. 6). In contrast to the findings with propionate, the expression level of prpD in valerate supplemented with vitamin B₁₂ was not significantly different from that observed in 7H9 broth (Fig. 6). Importantly, the induction of the prpDC operon in *M. tuberculosis* grown in propionate relative to that in valerate suggests the preferential routing of propionyl-CoA through the methylcitrate cycle when *M. tuberculosis* is cultured in propionate and is consistent with the observed delay in the growth of the organism on this carbon source as a result of the accumulation of toxic methylcitrate cycle intermediates (Fig. 3A).

**DISCUSSION**

The metabolic capacity of pathogens during infection is a key factor in defining the interaction with the host. Where pathogenesis is obligate, as is the case with *M. tuberculosis*, metabolism is integral to the ability of the organism to infect, survive, and be transmitted and therefore cannot be separated from concepts of virulence. Propionyl-CoA, as a precursor in several lipid biosynthetic pathways, including those for the...
production of the virulence factors sulfolipid 1 and phthiocerol dimycolate as well as the terminal product of the β-oxidation cycle, effectively encapsulates this notion, providing a natural intersection for virulence and central carbon metabolism. In this study, we established the capacity of the vitamin B12-dependent methylmalonyl pathway to fulfill a key role in propionate metabolism during the growth of the organism on fatty acids of odd chain length. Specifically, we presented genetic evidence that mutAB encodes a functional vitamin B12-dependent MCM and that an active methylmalonyl pathway enables the utilization of propionate as the sole carbon source in the absence of both the glyoxylate and methylcitrate cycles. The differential growth kinetics on propionate of a prpDC mutant strain versus H37Rv in the presence of the ICL inhibitor 3NP illustrated the effect that toxic intermediates of the methylcitrate cycle may have on the growth phenotype of M. tuberculosis in vivo (34, 36, 50), even if vitamin B12 supplementation improved the growth of the mutant strain further unscorched the anaplerotic contribution of the methylmalonyl pathway to the growth of M. tuberculosis on this substrate.

In contrast, the methylmalonyl pathway was entirely dispensable for the growth of H37Rv on heptadecanoate, the β-oxidation of which yields seven molecules of acetyl-CoA for each molecule of propionyl-CoA. Although vitamin B12 supplementation improved the growth of the prpDC mutant on heptadecanoate, it is important to note that this strain was nonetheless able to grow on this carbon source in the absence of vitamin B12. This suggests that the relative abundance of acetyl-CoA available to support cell growth and division allows small amounts of derivative propionyl-CoA to be assimilated into cellular lipids, thus reducing the toxic buildup of propionyl-CoA in the methylcitrate cycle-defective prpDC mutant. Previously, in demonstrating the functionality of the vitamin B12-dependent methionine synthase MetH, we established the inability of M. tuberculosis to produce vitamin B12 in vitro in medium containing dextrose as the carbon source (56). Here, we have extended that observation to include fatty acids of odd chain length (C3, C5, and C17), reinforcing the need to supplement growth media with vitamin B12 if the contribution of vitamin B12-dependent pathways to M. tuberculosis metabolism is to be assessed in vitro. Importantly, the demonstrated functionality of MCM reiterates the potential relevance of vitamin B12 to mycobacterial pathogenesis. In this regard, it is interesting that the genome of the related mycobacterial pathogen Mycobacterium leprae encodes homologs of both MetH and MCM as well as MeaB (11). The M. leprae genome is the product of reductive evolution to an extent that it is considered to approximate a minimal mycobacterial gene set (11). The conservation of two vitamin B12-dependent enzymes therefore strongly implies a selective advantage associated with the retention of vitamin B12-dependent pathways (11). It also suggests that vitamin B12 is available in vivo, as unlike M. tuberculosis, the M. leprae genome has undergone wholesale decay in vitamin B12 biosynthetic genes but has retained intact vitamin B12 riboswitch regulatory motifs (46). Importantly, the possibility that M. tuberculosis is able to synthesize and/or access vitamin B12 in vivo could inform the apparently paradoxical dispensability of the methylcitrate cycle for the growth and persistence of M. tuberculosis Erdman in mice (37); that is, a functional methylmalonyl pathway might compensate for the loss of methylcitrate cycle activity, thereby enabling the replication (and persistence) of the prpDC mutant. This possibility, in turn, suggests that mutant strains might be profitably applied
as bioprobes to establish the availability of vitamin B$_2$$_y$ in vivo. These issues are currently under investigation in our laboratories.

The metabolic capacity of M. tuberculosis is a function of the environments encountered during parasitism of the human host (16) and therefore represents evolution from an environmental ancestor to a well-adapted intracellular pathogen. It is likely, therefore, that the conservation of vitamin B$_2$$_y$-dependent enzymes, in some cases, in addition to corresponding vitamin B$_2$$_y$-independent isoforms (12, 56), is indicative of the differential enzyme and cofactors required in heterogeneous in vivo environments. Although M. tuberculosis resides primarily within macrophages, accumulating evidence suggests that the number of cellular environments serving as potential habitats is probably diverse (19, 39, 53). ICL activity has been shown to be essential to the establishment of infection in the acute stage of tuberculosis in a murine infection model (36). Our finding that the methylmalonyl pathway can provide an anaplerotic feed to the TCA cycle raises the possibility that in addition to the carbon sources utilized, this essentiality may be dictated by the availability of vitamin B$_2$$_y$ in the initial stages of infection. The extents to which metabolic pathway and substrate utilizations are defined by the stage of infection, the tissue-specific distribution of nutrients, and the ability of the bacilli to access those nutrients therefore constitute fundamental aspects of mycobacterial pathogenesis that continue to demand elucidation. Is it significant, for example, that transcriptional events in mycobacterial pathogenesis that continue to demand elucidation are currently under investigation in our laboratory.

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