

Cholesterol Catabolism by *Mycobacterium tuberculosis* Requires Transcriptional and Metabolic Adaptations

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SUMMARY

To understand the adaptation of *Mycobacterium tuberculosis* to the intracellular environment, we used comprehensive metabolite profiling to identify the biochemical pathways utilized during growth on cholesterol, a critical carbon source during chronic infection. Metabolic alterations observed during cholesterol catabolism centered on propionyl-CoA and pyruvate pools. Consequently, growth on this substrate required the transcriptional induction of the propionyl-CoA-assimilating methylcitrate cycle (MCC) enzymes, via the Rv1129c regulatory protein. We show that both Rv1129c and the MCC enzymes are required for intracellular growth in macrophages and that the growth defect of MCC mutants is largely attributable to the degradation of host-derived cholesterol. Together, these observations define a coordinated transcriptional and metabolic adaptation that is required for scavenging carbon during intracellular growth.

INTRODUCTION

The physiological state of intracellular pathogens is inextricably linked to the host cell in which they reside, and each pathogen is metabolically adapted to its specific niche. Nutrition is a primary factor driving this adaptation, as each pathogen is forced to subsist on locally available host-derived compounds. Intraphagosomal pathogens, like *Mycobacterium tuberculosis*, must acquire nutrients while surrounded by a host-derived membrane (Appelberg, 2006). Perhaps because of this sequestration, *M. tuberculosis* is thought to utilize these surrounding host lipids as carbon sources (Russell et al., 2010).

The preferential use of lipid carbon sources by *M. tuberculosis* in vivo has been inferred from the transcriptional induction of genes encoding fatty acid degradation enzymes (Schnappinger et al., 2003) and from the requirement for specific pathways in central carbon metabolism (Marrero et al., 2010; McKinney

et al., 2000) that are often essential for the utilization of nonglycolytic carbon sources. The identity of specific host lipids used by *M. tuberculosis* remained elusive until a dedicated cholesterol uptake and catabolic pathway was identified in the bacterium (Van der Geize et al., 2007). The subsequent demonstration that both cholesterol uptake and degradation are necessary for growth and survival of *M. tuberculosis* in chronically infected mice (Nesbitt et al., 2010; Pandey and Sassetti, 2008) verified that this single component of host membranes is an essential nutrient during chronic infection.

Despite these observations, it remained unclear whether cholesterol catabolism contributes significantly to the central metabolic requirements for growth in the complex host environment. Although cholesterol is catabolized to some extent throughout infection (Chang et al., 2009), cholesterol uptake mutants grow normally before the onset of adaptive immunity in mice and in the resting macrophages that characterize this stage of infection (Pandey and Sassetti, 2008). These observations indicate that additional carbon sources are available to the bacterium in vivo, such as fatty acids, sugars, and amino acids. In addition, environmental conditions encountered during infection, including altered O₂, CO, NO, and H⁺ concentrations (Chan et al., 1995; Kumar et al., 2008; Rohde et al., 2007; Via et al., 2008), could contribute to metabolic alterations. To begin to understand the specific role of nutrition in the overall metabolic adaptation to the host, we sought to understand how the only defined in vivo carbon source, cholesterol, influenced the physiology of the bacterium during infection.

To do this, we used comprehensive metabolite profiling to characterize the metabolic state of *M. tuberculosis* during growth on defined carbon sources. Utilization of cholesterol profoundly altered the abundance of a variety of primary metabolites, most notably the intermediates of a propionyl-CoA catabolic pathway known as the methylcitrate cycle (MCC). The activity of this pathway appeared to be rate limiting for growth under these conditions, as transcriptional induction of the dedicated enzymatic steps was essential. While propionyl-CoA can be derived from a variety of host components other than cholesterol, we demonstrate that the requirement for this metabolic pathway in vivo is largely attributable to the utilization of this single host carbon source.

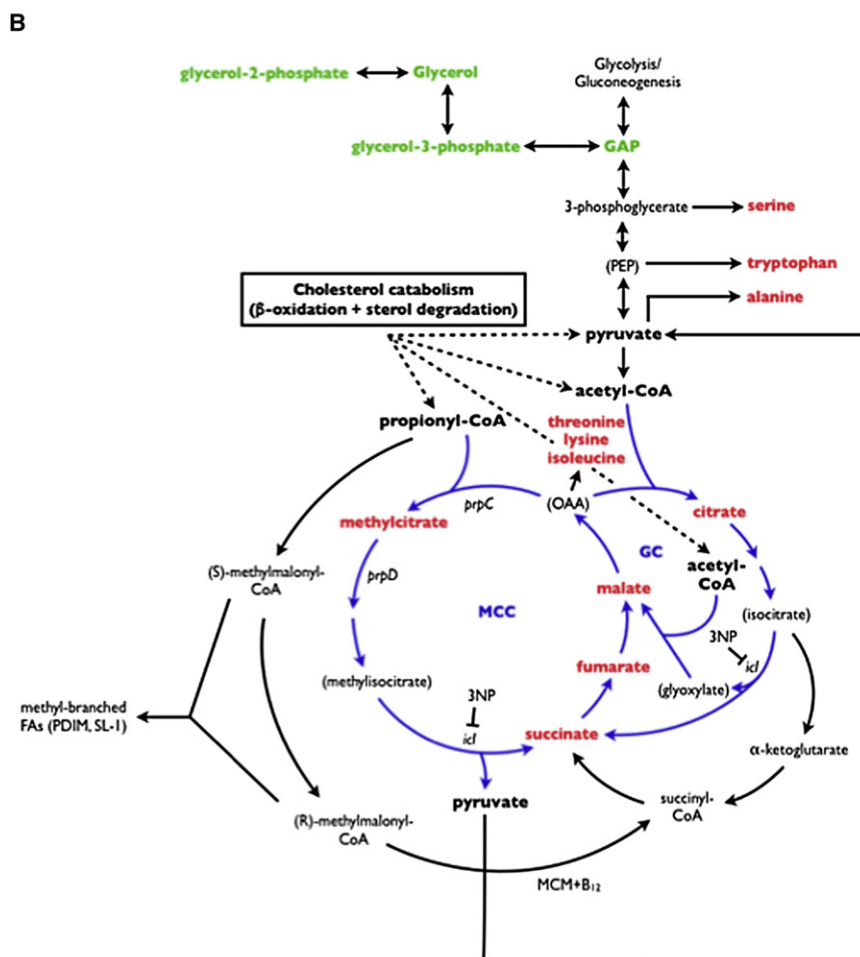
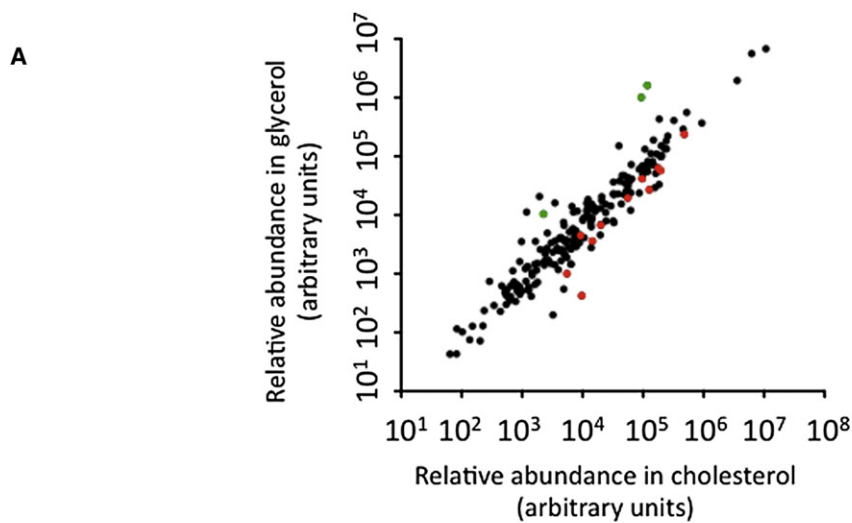


Figure 1. Growth on Cholesterol Causes Metabolic Changes Consistent with Increased MCC Flux

Quadruplicate samples of *Mtb* were grown with either cholesterol or glycerol as primary carbon sources, and LC/MS and GC/MS were used to quantify the relative abundance of primary metabolites.

(A) The average relative abundance of each metabolite in glycerol- or cholesterol-grown bacteria is plotted. Subsets of the differentially abundant metabolites are shown in red or green to represent their accumulation in either cholesterol- or glycerol-fed cultures, respectively. These metabolites are highlighted in the same colors in (B) and described in Table 1.

(B) Differentially represented metabolites indicate increased MCC flux and are shown in relation to the relevant carbon metabolic pathways. Both the MCC and the glyoxylate cycle (GC) are highlighted in blue. The predicted catabolism of cholesterol into acetyl-CoA, propionyl-CoA, and pyruvate (Van der Geize et al., 2007) is indicated. The B12-dependent methylmalonyl CoA mutase (MCM) is also shown. See Tables S1 and S2 for details regarding metabolite quantification and identification, respectively.

elucidated, but the catabolism of this compound is predicted to produce an ill-defined mixture of propionyl-CoA, acetyl-CoA, and pyruvate (Van der Geize et al., 2007). To characterize the central metabolic consequences of growth on this sterol, *M. tuberculosis* was grown in minimal media containing either cholesterol or glycerol, carbon sources that are initially catabolized by distinct pathways (Figure 1). Liquid- and gas-chromatography-coupled mass spectrometry was then used to globally profile metabolite pools in each population. Individual metabolites were identified by comparison to a library of retention times and masses of authentic standards, and the relative abundance of each metabolite was determined with respect to carbon source.

This analysis unambiguously identified 146 distinct metabolites, which were distributed throughout amino acid, carbohydrate, lipid, nucleotide, cofactor, and central carbon metabolism (Table S1 available online). Overall, metabolite levels were comparable between

RESULTS

Metabolomic Profile of Cholesterol Catabolism

The dedicated pathway responsible for the degradation of cholesterol into primary metabolites remains to be completely

these two cultures (Figure 1A), which is consistent with the similar doubling times observed in these media (Pandey and Sassetti, 2008). Statistical analysis of these data identified 29 metabolites that accumulated to a significant degree in the cholesterol-grown cells. (Table 1 and Table S1). Among these

Table 1. Metabolites that Significantly Accumulated following Growth on Cholesterol

Biochemical Name	Cholesterol/ Glycerol Ratio ^a	p Value
Tryptophan	2.07	0.0226
2-Amino adipate	5.54	0.0022
Alanine	2.02	0.027
Beta-alanine	4.44	0.0098
Lysine	3.39	0.0424
Pipecolate	2.15	0.023
S-adenosylhomocysteine (SAH)	1.85	0.0432
Threonine	2.86	0.0218
Isoleucine	2.33	0.0376
Methylsuccinate	16.28	<0.001
Histidine	2.9	0.0195
N-acetylmethionine	2.19	0.0079
Serine	2.81	0.0259
Gamma-glutamylthreonine	1.9	0.0205
Adenosine-5'-diphosphoglucose	3.45	0.0103
Mannose	4.02	0.0494
Mannose-1-phosphate	5.34	0.0125
Trehalose 6-phosphate	5.17	0.0224
2-Methylcitrate	22.81	<0.001
Citrate	4.02	0.0054
Fumarate	2.95	0.0371
Malate	5.49	0.012
Succinate	4.67	0.0022
Adenosine 5'-monophosphate (AMP)	1.73	0.0305
Thymidine 5'-monophosphate	3.38	0.0294
Pantothenate	5	0.0024
Nicotinate	3.66	0.0264
Nicotinate ribonucleoside	2.91	0.0338
Nicotinic acid mononucleotide (NaMN)	2.2	0.0011

^aThis column represents the relative abundance of each species in the indicated media. The p values were calculated as described in Experimental Procedures.

compounds were malate, fumarate, succinate, and methylcitrate. These compounds account for four of the six intermediates of the MCC, a variant of the tricarboxylic acid cycle used for the conversion of propionyl-CoA into pyruvate (Pronk et al., 1994; Textor et al., 1997). The remaining two intermediates were not detected under either condition. Consistent with increased activity of the MCC, we also found an accumulation of amino acids derived from the MCC intermediate oxaloacetate (OAA) and the MCC product, pyruvate (Figure 1B). Pyruvate may also be directly liberated from the cholesterol steroid nucleus during catabolism (Van der Geize et al., 2007), further increasing the abundance of this compound. This analysis suggests that propionyl-CoA and pyruvate metabolism are central for the adaptation of *M. tuberculosis* to growth on cholesterol, and these metabolites are linked via the MCC.

Propionyl-CoA Is Liberated from the Side Chain of Cholesterol

In addition to fueling the MCC, propionyl-CoA can also be used as a precursor for methyl branched-chain lipid synthesis after conversion to methylmalonyl CoA (Jain et al., 2007). We have previously shown that carbon derived from the branched side chain of cholesterol was efficiently incorporated into mycobacterial lipids (Pandey and Sassetti, 2008), making the side chain terminus a likely source of propionyl-CoA. To directly investigate the metabolic fate of this moiety, we analyzed the composition of the cellular lipids labeled after growth in cholesterol containing a ¹⁴C label at the terminus of its side chain (Figure 2A). Upon profiling the total polar lipid extract, we found that a single species incorporated more than 80% of the ¹⁴C derived from [26-¹⁴C]-cholesterol, in contrast to [1,2-¹⁴C]-acetate, which labeled a variety of other lipids. Mass spectrometry was used to identify this species as sulfolipid-1 (SL-1), one of the prominent methyl branched-chain lipids of *M. tuberculosis* (Figure 2). Another major branched-chain lipid previously shown to incorporate propionate-derived carbon, phthiocerol dimycocerosate, was not present in this extract.

Because both the abundance and chain length of SL-1 are directly related to availability of the propionyl-CoA precursor (Jain et al., 2007), we used these metrics to estimate the abundance of this precursor metabolite in cholesterol-grown cells. We found that both the molecular weight of SL-1 and its relative abundance, compared to other unrelated ions in the mass spectrum, increased in cholesterol-grown cultures (Figure 2C). This observation was consistent with the cholesterol-induced increase in length and abundance of phthiocerol dimycocerosate (Yang et al., 2009; data not shown), which is synthesized from the same precursor pool. The preferential flux of cholesterol side-chain-derived carbon into SL-1 and the inferred increase in methylmalonyl CoA in cholesterol-grown cells both indicate that cholesterol is a significant source of propionyl-CoA and that at least a portion of this metabolite is derived from the side chain terminus.

Functional MCC Enzymes Are Required for Growth in the Presence of Cholesterol

To determine the importance of propionyl-CoA metabolism during growth on cholesterol, we tested the ability of MCC-deficient mutants to grow using this carbon source. We found that the deletion of the *prpC* and *prpD* genes, encoding the two dedicated steps of this pathway, specifically impaired the bacterium's ability to grow in media containing cholesterol as a primary carbon source (Figures 3A and 3B). Similarly, deletion of the *icl1* gene, which encodes a bifunctional methylisocitrate lyase and isocitrate lyase (MCL/ICL) that is necessary for both the MCC and glyoxylate cycles (Gould et al., 2006), caused a specific growth defect in cholesterol-containing media (Figures 3E and 3F). Deletion of the partially redundant *icl2* gene, either alone or in combination with the *icl1* mutation, had a more modest effect.

In many bacteria, including mycobacteria, the inability to assimilate propionyl-CoA inhibits growth even in the presence of other carbon sources (Muñoz-Eliás et al., 2006; Savvi et al., 2008). This is likely due to the accumulation of intermediates that inhibit other essential pathways (Rocco and Escalante-Semerena, 2010). Indeed, when we cultured $\Delta prpDC$ or $\Delta icl1$

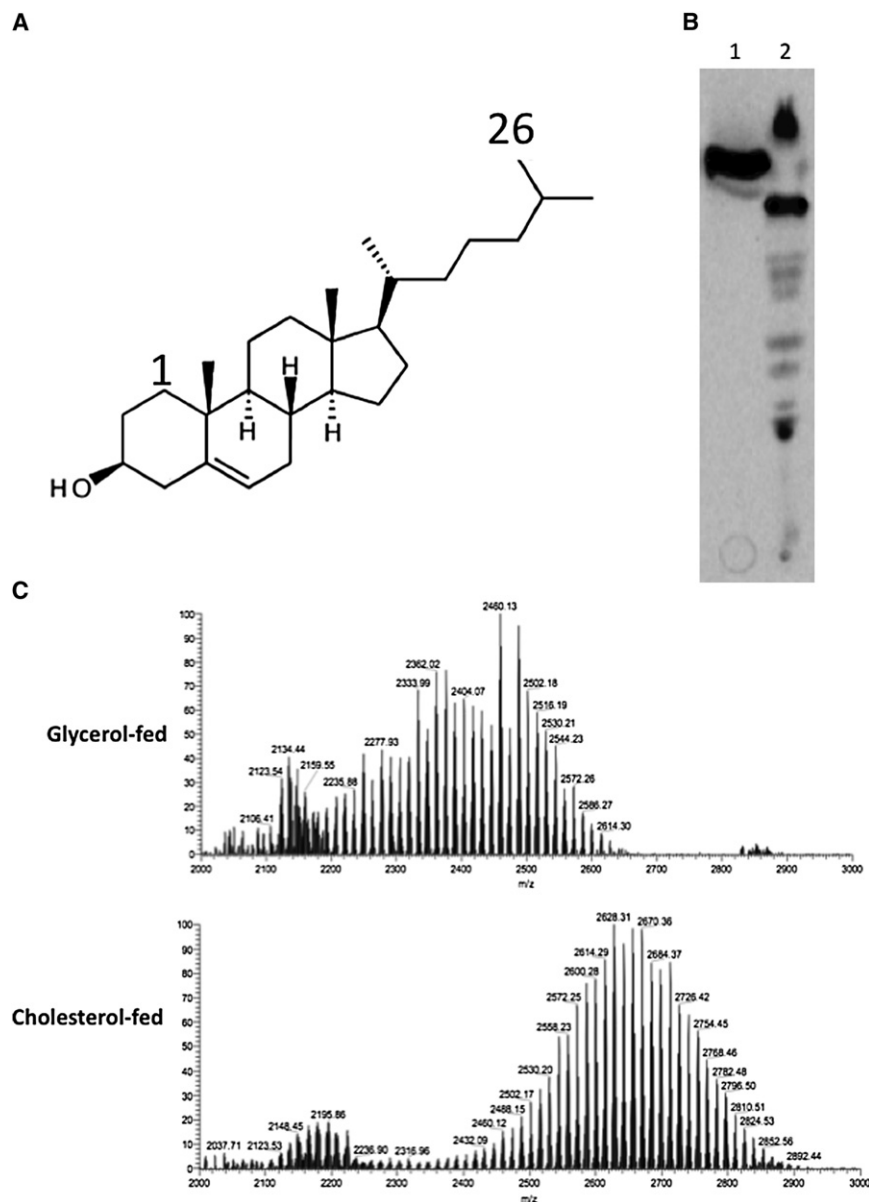


Figure 2. Cholesterol Side-Chain-Derived Propionate Is Incorporated into SL-1 and Increases the Mass of This Lipid

(A) The cholesterol molecule. Carbon 26 is indicated.

(B) Total polar lipids were extracted from [26-¹⁴C] cholesterol-fed (lane 1) and [1,2-¹⁴C]acetate-fed (lane 2) bacteria, separated by thin layer chromatography, and detected by autoradiography.

(C) The major species labeled by cholesterol was isolated and identified as SL-1 using ESI-TOF MS. The *m/z* peaks depicted match the previously described lipofoms of SL-1 (Kumar et al., 2007). TLC-purified samples of SL-1 from cholesterol-fed and from glycerol-fed bacteria were compared to demonstrate that growth on cholesterol increased the mass of SL-1.

in another tricarboxylic acid cycle variant, the glyoxylate shunt. We conclude that propionyl-CoA metabolism is essential for growth in the presence of cholesterol.

Transcriptional Induction of MCC Genes by Rv1129c Is Required for Propionyl-CoA Metabolism In Vitro

The accumulation of MCC intermediates during the growth of *M. tuberculosis* on cholesterol suggested that the activity of the dedicated MCC enzymes may be increased under these conditions. Indeed, we found that the abundance of the mRNA encoding both *prpC* and *prpD* was increased fivefold in cholesterol-grown cultures (Figure 4). The *rv1129c* gene appeared to be divergently transcribed from the *prpDC* operon and encoded a protein that was 47% identical to RamB, the regulator of *icl* gene transcription in actinobacteria (Gerstmeier et al., 2004; Micklinghoff et al., 2009). This suggested that Rv1129c may perform a function similar to that of the

mutants in mixed-carbon-source media containing cholesterol, we observed growth defects that were comparable to the single-carbon-source media (Figures 3C and 3G). To verify that this growth defect was due to propionyl-CoA-related toxicity, we enabled an alternative assimilation system, the methylmalonyl pathway (MMP). This pathway converts propionyl-CoA into succinyl-CoA via the B12-dependent methylmalonyl CoA mutase (MCM) enzyme (Figure 1B), allowing MCC mutants to grow on propionyl-CoA-generating substrates (Savvi et al., 2008). Because *M. tuberculosis* is unable to synthesize B12 under these conditions, the MMP is only active upon B12 supplementation. As anticipated, the addition of B12 to these mixed-carbon-source cultures reversed the growth defect imparted by MCC mutations (Figures 3D and 3H). The modest residual defect of the $\Delta icl1$ mutant in media containing only cholesterol may be attributable to the additional role played by this enzyme

homologous RamB and regulate the transcription of *prpDC*. To investigate this, we compared the level of *prpDC* mRNA in wild-type bacteria with a strain in which the *rv1129c* gene had been deleted. We found that transcript levels of both *prpD* and *prpC* were dramatically reduced in the absence of *rv1129c*, regardless of carbon source, and that this phenotype could be complemented in trans by expressing a wild-type copy of the gene (Figure 4B).

To understand the physiological relevance of this regulation, we determined if the deletion of *rv1129c* would alter growth on propionyl-CoA-generating substrates. We found that the *rv1129c* mutant was unable to grow using either propionate or cholesterol as a primary carbon source (Figures 4C and 4E). This phenotype was rescued either by genetic complementation or by adding B12 to circumvent the MCC requirement (Figures 4D and 4F), demonstrating that transcriptional induction of

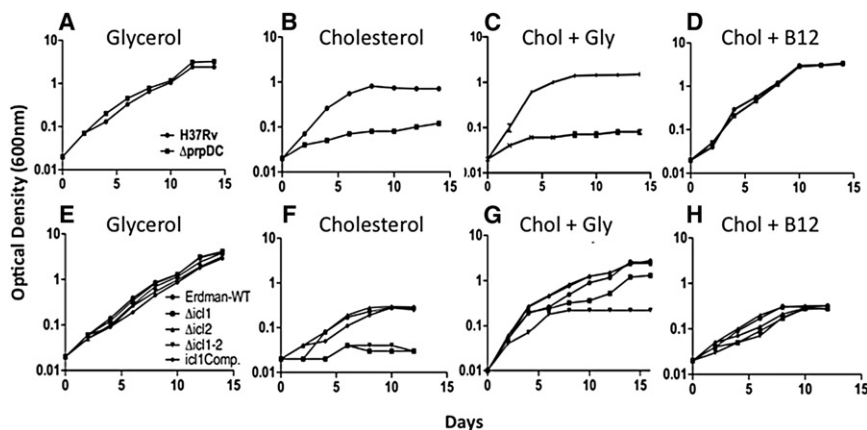


Figure 3. The MCC Is Required for the Metabolism of Cholesterol-Derived Propionate

The indicated glycerol mutants were grown in the presence of 0.1% glycerol (A and E), 0.01% cholesterol (B and F), or 0.01% cholesterol and 0.1% glycerol (C and G) as primary carbon sources. Vitamin B12 supplementation (D and H) allowed all mutants to grow using cholesterol. Optical density was measured at the indicated time points and is plotted on a log scale. *prpDC* mutants were generated in the H37Rv strain, and *icl1/2* mutants were derived from the Erdman strain.

prpDC via Rv1129c is required for the assimilation of cholesterol-derived propionate.

Cholesterol Is a Significant Source of the Propionyl-CoA Generated during Intracellular Growth

The dedicated MCC components PrpD and PrpC, as well as the multifunctional MCL/ICL enzymes, are required for intracellular growth in macrophages (Muñoz-Elías and McKinney, 2005; Muñoz-Elías et al., 2006), indicating that propionyl-CoA metabolism is critical in this niche. To determine if this requirement could be attributed directly to cholesterol catabolism, we investigated the contribution of cholesterol-derived propionyl-CoA to this phenotype.

First, we sought to determine whether the previously described intracellular growth defect of MCC mutants was due to propionyl-CoA-related toxicity. To do this, we infected bone-marrow-derived macrophages (BMDMs) with *M. tuberculosis* and inhibited the MCC by genetic deletion of *prpDC* or chemical inhibition of MCL/ICL activity with 3-nitropropionate (3-NP) (Schloss and Cleland, 1982). Both MCC inhibition strategies resulted in a significant inhibition of intracellular growth. Addition of B12 to enable the alternative MMP caused a dose-dependent reversal of both phenotypes (Figures 5A and 5B), indicating that propionyl-CoA-related toxicity was the basis for these growth defects. Similarly, we found that the *rv1129c* regulatory mutant was unable to grow in BMDM unless supplemented with B12, indicating that this protein contributes to intracellular growth largely by inducing the expression of MCC enzymes (Figure 5C).

We then investigated whether cholesterol contributed significantly to the bacterial propionyl-CoA pool during intracellular growth. To do this, we used *M. tuberculosis* mutants lacking the Mce4 cholesterol uptake system, which imports cholesterol at a greatly reduced rate but grows normally in resting BMDM (Mohn et al., 2008; Pandey and Sassetti, 2008). To estimate the propionyl-CoA pool in wild-type and *mce4* mutants during intracellular growth, we compared the relative sensitivity of these strains to Icl1/2 inhibition with 3-NP. The cholesterol uptake mutants were significantly more resistant to 3-NP, indicating that less MCL/ICL activity was necessary and, therefore, that less propionyl-CoA was generated in the absence of cholesterol catabolism (Figure 5D). To further support this conclusion, we determined if the *mce4* deletion was also able to suppress the growth defect of the $\Delta prpDC$ mutant. Indeed, we observed

that $\Delta mce4/\Delta prpDC$ double mutants grew significantly more rapidly than $\Delta prpDC$ single mutants in BMDM, and that the residual growth defect of the double mutants could be fully reversed by the addition of B12 (Figure 5E). We conclude that MCC mutants are unable to grow intracellularly because of propionyl-CoA toxicity, and a significant fraction of this metabolite is produced via the catabolism of host cholesterol.

DISCUSSION

Many approaches have been used to determine how the intracellular environment influences the physiological state of a pathogen during infection. Most identify biochemical pathways that are expressed or are essential during infection, and this information is used to infer both the metabolic state of the bacterium and the environment that imposes this state. However, the complexity of metabolic networks makes these inferences difficult, as a single enzyme can participate in multiple biochemical pathways, and the reliance on any pathway can be influenced by a number of different environmental factors. In this work, we used a unique combination of direct metabolomic profiling and genetic epistasis analysis to functionally connect a distinct feature of the intracellular environment with its metabolic consequence in the pathogen.

To understand the central metabolic adaptations required for the utilization of the host-derived carbon source, cholesterol, we globally surveyed the steady-state levels of primary metabolites during growth on this compound. This approach is fundamentally different from more commonly used profiling of mRNA or protein levels, which may not correlate with the metabolite levels (Jozefczuk et al., 2010). We found that the differential utilization of a pathway could be predicted by the abundance of both its specific intermediates and its more stable biosynthetic products. Labeling of proteinogenic amino acids has been used extensively to predict the flux through central carbon metabolism (Zamboni et al., 2009), and our data suggest that pool sizes of individual amino acids are also likely stable enough to serve this purpose. Together, these data provided a holistic view of cellular metabolism and allowed us to identify pathways that were critical for cholesterol catabolism.

The relatively dramatic accumulation of the PrpD substrate, methylcitrate, suggested that the expression of this enzyme might be insufficient for the flux of propionyl-CoA derived from

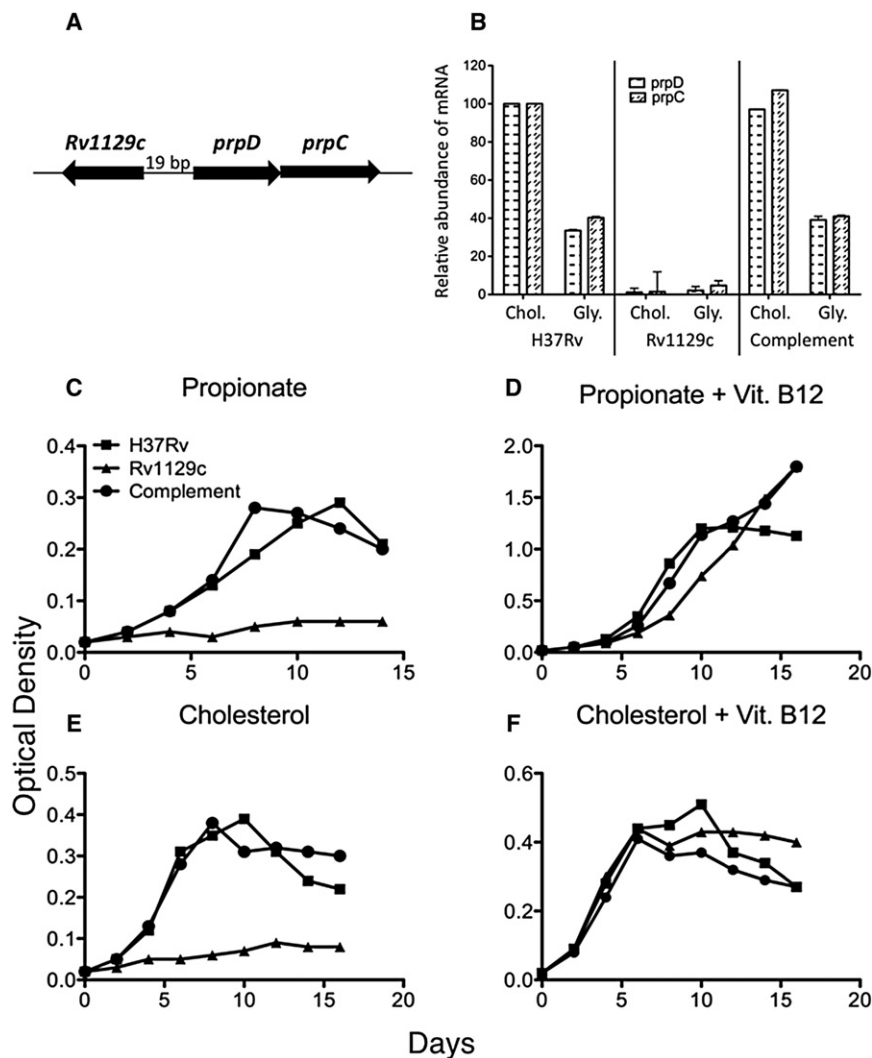


Figure 4. The Transcriptional Activation of MCC Genes by Rv1129c Is Required for Growth on Cholesterol

(A) Genomic organization of *rv1129c* and the *prpDC* genes.

(B) Rv1129c was required for both basal *prpDC* expression and for the induction of these genes in cholesterol media. Quantitative PCR was used to measure the relative abundance of *prpD* and *prpC* mRNA after 48 hr of growth in either 0.01% cholesterol or 0.1% glycerol as primary carbon sources. Error bars indicate standard deviation of triplicate samples.

(C–F) Rv1129c is required for growth on propionyl-CoA-generating substrates. Bacteria were cultured in the indicated defined primary carbon sources with or without vitamin B12, and growth was monitored by optical density. Data are representative of three independent experiments.

proteins to control the pathways necessary for propionyl- and acetyl-CoA catabolism, and differential expression of these systems could be mediated via distinct allosteric regulation.

Consistent with previous observations (Muñoz-Elias et al., 2006; Savvi et al., 2008), we found that the growth of mutants lacking sufficient MCC activity was inhibited by propionate, even in the presence of an additional carbon source. Several mechanisms have been proposed to mediate propionyl-CoA-related toxicity. For example, this metabolite has been shown to inhibit pyruvate dehydrogenase (PDH) and citrate synthase in other bacteria (Brock and Buckel, 2004; Man et al., 1995; Maruyama and Kitamura, 1985).

While the accumulation of glycolytic intermediates we observed in cholesterol-grown mycobacteria could be consistent with these mechanisms, it is unclear whether the unusual PDH complex (Tian et al., 2005) or the apparently redundant citrate synthase enzymes (CitA and GltA) of mycobacteria are inhibited by propionyl-CoA. Similarly, propionate-related toxicity in MCC-deficient *Salmonella* is due to the accumulation of a specific 2-methylcitrate isomer that inhibits the gluconeogenic enzyme, fructose 1,6 biphosphatase (Rocco and Escalante-Semerena, 2010). However, the type II fructose biphosphatase expressed by *Mtb* bears little resemblance to the *Salmonella* enzyme. Thus, while abundant examples of this phenomenon have been reported, it is currently unclear whether these previously described mechanisms are relevant in mycobacteria.

These metabolic insights provided the information necessary to test whether the MCC requirement during intracellular growth was due to host cholesterol utilization. The ability of *mce4* mutation to alleviate the intracellular growth defect produced by either *prpDC* mutation or MCL/ICL inhibition implicates cholesterol as a major source of propionate in vivo, even under conditions in

cholesterol. Indeed, it appears that several propionate units are liberated from each cholesterol molecule. In addition to the terminal side-chain carbons, at least two other propionyl-CoA molecules may be produced (Van der Geize et al., 2007), suggesting that as much as one third of the carbon derived from cholesterol could enter the MCC. While cholesterol appears to be a significant source of propionyl-CoA, the incomplete suppression of the $\Delta prpDC$ mutant's intracellular growth defect by *Mce4* mutation indicates that either *Mce4*-independent cholesterol uptake pathways operate in this environment or that alternative carbon sources, such as odd-chain fatty acids or branched-chain amino acids, also contribute to the propionyl-CoA pool during infection.

The increased reliance on the MCC pathway during cholesterol catabolism was confirmed by demonstrating that transcriptional induction of the *prpDC* genes via the Rv1129c protein was required for growth. The DNA-binding function of the homologous RamB protein of *Corynebacterium glutamicum* is post-translationally regulated (Cramer et al., 2007; Micklinghoff et al., 2009), perhaps via binding of an intermediary metabolite. Thus, mycobacteria appear to use very similar regulatory

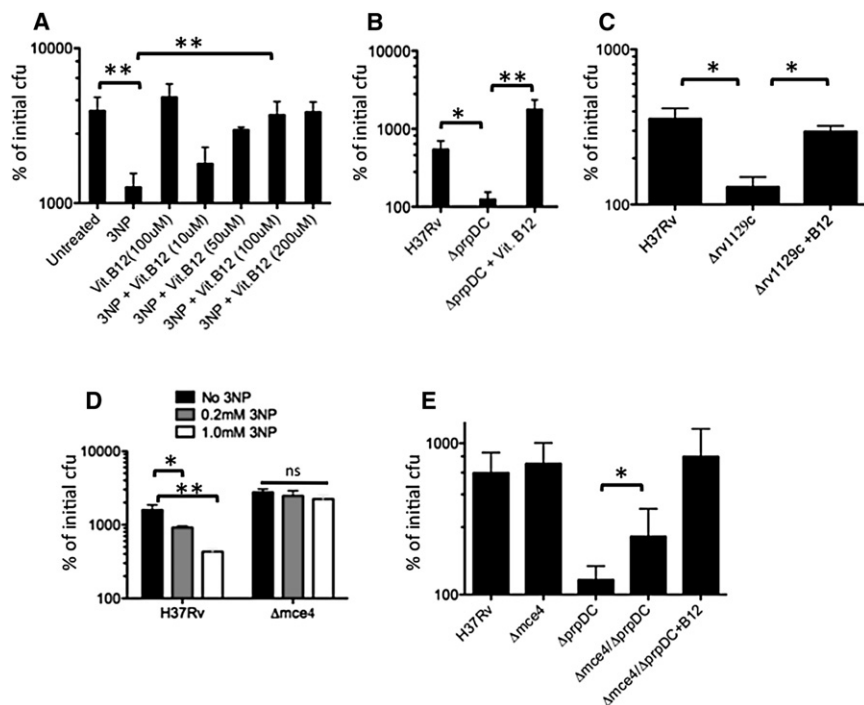


Figure 5. Cholesterol Is an Intracellular Source of Propionate

(A and B) MCC activity is required for propionyl-CoA metabolism during intracellular growth. BMDM were infected with H37Rv or the indicated mutant strains. The MCC was inhibited either by addition of 3NP to block MCL/ICL activity (A) or by genetic deletion of the *prpDC* genes (B). Vitamin B12 was added to enable the MMP. The number of intracellular bacteria at Day 6 postinfection are plotted relative to the number of initial cell-associated colony-forming units (cfu).

(C) Transcriptional activation of *prpDC* is required for intracellular growth. BMDM were infected with H37Rv or the $\Delta 1129c$ mutant, and the cfu present on Day 6 are presented as in (A).

(D) *Mce4* mutants are relatively resistant to 3NP treatment during intracellular growth. BMDM were infected with H37Rv or $\Delta mce4$ mutant bacteria in the presence or absence of the indicated doses of 3NP. Intracellular cfu at Day 6 are plotted.

(E) *Mce4* mutation partially suppresses the intracellular growth defect of the $\Delta prpDC$ mutant. The cfu detected after 6 days of intracellular growth are presented. Each experiment in this figure was performed at least twice. Representative experiments are shown in (A–D), and error bars indicate standard deviation of triplicate or quadruplicate samples. (E) represents the average of three independent experiments. Statistical significance was calculated by Student's *t* test. ns indicates $p > .05$. * $p < .05$. ** $p < .005$.

which cholesterol is not absolutely required for growth. This MCC mutant phenotype was reminiscent of strains lacking either the *hsaC* gene (Yam et al., 2009) or the “*igr*” locus (Chang et al., 2007; Chang et al., 2009) that encode functions dedicated to earlier steps in cholesterol degradation. Like MCC mutants, these strains are intoxicated in the presence of cholesterol and are unable to grow in resting macrophages or acutely infected animals. Together, these observations strongly argue that *M. tuberculosis* obtains a significant amount of its carbon requirements from cholesterol.

In contrast to their essentiality during intracellular growth in macrophages, the *prpDC* genes have been found to be dispensable for bacterial growth in the mouse model of tuberculosis (TB) (Muñoz-Elías et al., 2006). This observation was particularly surprising, as cholesterol catabolism occurs throughout infection (Chang et al., 2009; Pandey and Sassetti, 2008; Yam et al., 2009), and cell wall lipid alterations consistent with propionyl-CoA assimilation are evident in bacteria isolated from mouse tissue (Jain et al., 2007). This apparent contradiction could be explained if the B12-dependent MMP was active under these conditions, either because the bacterium acquires B12 from the host or because some aspect of the host environment stimulates B12 production by the pathogen (Savvi et al., 2008). Our observation that B12 supplementation reverses the growth defect of *prpDC* mutants in macrophages supports this hypothesis.

This work highlights propionyl-CoA assimilation as a critical feature of *M. tuberculosis* metabolism in vivo and demonstrates that this requirement is a direct result of the nutritional environment in the mycobacterial phagosome. However, even within

this relatively isolated compartment, the bacterium is likely to catabolize multiple carbon sources (de Carvalho et al., 2010), and inhibiting the bacterium's ability to utilize individual nutrients is unlikely to represent a fruitful therapeutic strategy. In contrast, inhibition of at least three distinct steps in cholesterol catabolism (*hsaC*, *igr*, or MCC-encoding genes) results in metabolic toxicity that might be effectively exploited to treat TB. Thus, a fundamental understanding of mycobacterial physiology during infection is critical for identifying vulnerabilities that can be used for the rational design of more effective TB therapies.

SIGNIFICANCE

***Mycobacterium tuberculosis* replicates within a phagosome-like compartment of the host macrophage, an environment containing limited nutrients, low pH, and abundant oxidative radicals. A number of central metabolic pathways have been implicated in the adaptation to this niche, but the basis for these requirements remained obscure. To understand which of these metabolic adaptations could be attributed directly to the unique nutritional environment of the phagosome, we identified metabolite pools that were specifically altered during growth on cholesterol, a critical carbon source during chronic infection. The most significant metabolite perturbation was the dramatic accumulation of methylcitrate cycle (MCC) intermediates. We show that these intermediates are produced during the catabolism of propionyl-CoA derived from the terminal carbons of the cholesterol side chain. Consistent with a requirement for elevated MCC activity, growth on cholesterol required the**

transcriptional induction of two MCC enzyme-encoding genes, a function mediated by the Rv1129c protein. Using a combination of genetic and chemical-genetic epistasis analyses, we found that both Rv1129c and the MCC enzymes were required for propionyl-CoA metabolism during intracellular growth in macrophages and that a portion of this metabolite was derived directly from cholesterol. Thus, *M. tuberculosis* appears to satisfy a significant fraction of its carbon requirement by scavenging host cholesterol, and a coordinated transcriptional and metabolic adaptation is necessary for the assimilation of propionyl-CoA liberated by the catabolism of this compound.

EXPERIMENTAL PROCEDURES

Growth and Maintenance of Bacterial Strains

M. tuberculosis was cultured in complete Middlebrook 7H9 medium containing 0.05% Tween 80 and oleic acid-albumin-dextrose-catalase supplement. Kanamycin, Hygromycin B, and Zeocin were used at 25, 50, and 25 $\mu\text{g/ml}$, respectively. For cholesterol experiments, bacteria were grown in minimal media containing asparagine (0.5 g/l), KH_2PO_4 (1.0 g/l), Na_2HPO_4 (2.5 g/l), ferric ammonium citrate (50 mg/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g/l), CaCl_2 (0.5 g/l), ZnSO_4 (0.1 mg/l), 0.2% tyloxapol, 0.2% ethanol, and either 0.1% glycerol or 0.01% cholesterol. Where noted, Vitamin B₁₂ (cyanocobalamin; Sigma) was added to a final concentration of 10 $\mu\text{g/ml}$. Growth was monitored by optical density at 600 nm.

Genetic Manipulation of Strains

$\Delta rv1129c$ was generated in *M. tuberculosis* H37Rv by “recombineering” as described elsewhere (van Kessel and Hatfull, 2007), except the recombination functions were expressed from the pNIT::ET plasmid (GenBank No. GU459073). Briefly, phage Che9c recombination genes were induced by overnight incubation with 10 μM isovaleronitrile. Bacteria were then electroporated with a linear substrate containing 500 bp of homology to both the upstream and downstream regions of *rv1129c* flanking a hygromycin-resistance cassette. The presence of both recombination junctions were verified by PCR and sequencing. The absence of the *rv1129c* open reading frame was also confirmed by PCR. Nucleotides 1253102–1254505 were deleted from the genome. A complemented strain was generated by transforming the mutant with pJEB402 (Guinn et al., 2004) containing full-length Rv1129c sequence (nucleotides 1253074–1254564 in the H37Rv sequence) cloned between HindIII and KpnI sites. This plasmid integrates in single copy into the phage L5 *attB* site (Lee et al., 1991). The construction of *icl1/2* mutants in *M. tuberculosis* strain Erdman was described in (Muñoz-Eliás and McKinney, 2005). *prpDC* mutants were generated in H37Rv or an $\Delta mce4$ mutant of H37Rv (Joshi et al., 2006), as described in (Muñoz-Eliás et al., 2006).

Metabolomic Profiling

Strains were grown in quadruplicate in 50 ml of complete 7H9 medium until an optical density at 600 nm (OD_{600}) of 0.5 was reached. Cultures were then washed in PBS twice and resuspended in minimal media with either 0.01% cholesterol or 0.1% glycerol (Pandey and Sasseti, 2008) for 48 hr at 37°C. Cells were harvested at 4,000 g for 5 min at 4°C and lysed by vortexing in 10 ml chloroform:methanol (2:1). We transferred samples to glass vials dried under a stream of nitrogen and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis (in positive and negative ionization modes independently) using a Thermo-Finnigan LTQ mass spectrometer essentially as described elsewhere (Evans et al., 2009). We derivatized the same samples with bistrimethyl-silyl-trifluoroacetamide and subjected them to gas chromatography/mass spectrometry (GC/MS) analysis using a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer with electron impact ionization, as described by Lawton et al., 2008. All metabolite identities listed in Table S1 were assigned by comparison to a library of mass/charge (*m/z*), retention times, and MS/MS fragmentation spectra of authentic standards (Dehaven et al., 2010). GC/MS library spectra were also compared to databases maintained by the National Institute of Standards and Technology. All computational matches were confirmed by manual curation. Retention

times and mass spectra for each metabolite are listed in Table S2. To conservatively estimate the average relative abundance of metabolites that were undetectable in one sample, we created a “median scaled imputed” data set. In this transformation, the raw abundance value for each compound was divided by the median of its detected values. Both raw and scaled data sets are provided in Table S1. The ratios reported in Table S1 are based on scaled data. We calculated *p* values from scaled data using Welch’s *t* test.

Metabolic Labeling and Lipid Analysis

Logarithmically growing cultures of H37Rv were collected by centrifugation and resuspended in minimal medium containing 0.1 $\mu\text{Ci/ml}$ [$26\text{-}^{14}\text{C}$]cholesterol or [$1,2\text{-}^{14}\text{C}$]acetate (American Radiolabeled Chemicals, St. Louis, MO, USA). Labeling was performed for 16 hr. Cell pellets were washed twice with PBS and extracted with chloroform:methanol (2:1). Extracts were dried under nitrogen and resolved by thin layer chromatography using glass-baked silica gel plates (250 μm thick) using a chloroform:methanol:water (60:30:6) mobile phase. Radio-labeled species were detected with a phosphorimager. To identify and characterize SL-1, we excised the region of the TLC plate containing this species and subjected it to mass spectrometry in negative ion mode using a quadrupole time-of-flight (Q-ToF) mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source (Q-TOF Premier; Waters Corporation, Milford, MA, USA).

Macrophage Infections

We isolated BMDMs from C57BL/6 mice by culturing bone marrow cells in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 10% L929-conditioned medium, and 10 $\mu\text{g/ml}$ ciprofloxacin for 5 days. Animal manipulations were performed under the oversight of the University of Massachusetts Institutional Animal Care and Use Committee. Sixteen hours before infection, differentiated BMDMs were seeded on a 24-well tissue culture plate at 5×10^5 cells per well in the same medium lacking antibiotic. Macrophages were infected with different strains of *M. tuberculosis* at a multiplicity of infection of 1 for 4 hr at 37°C. Extracellular bacteria were removed by washing three times with warm PBS. Intracellular bacteria were quantified by lysing the cells with PBS containing 1% Triton X-100 at the indicated time points and plating dilutions on 7H10 agar. 3-NP or vitamin B12 was added at the indicated concentrations at the time of inoculation and maintained throughout the infection.

Quantitative PCR

Bacterial strains were grown in complete 7H9 medium until log phase, washed twice in PBS, and cultured in minimal medium with either 0.01% cholesterol or 0.1% glycerol for 48 hr at 37°C. Bacteria were collected by centrifugation, resuspended in Trizol, and lysed by bead beating. RNA was isolated according to the manufacturer’s directions and treated with DNase (TURBO DNA-free Kit; Applied Biosystems, Foster City, CA, USA). cDNA was generated with equal amounts of RNA from all samples (SMARTScribe Reverse Transcriptase; Clontech, Mountain View, CA, USA). We conducted quantitative PCR using iQ SYBR Green Supermix from BioRad and the following PCR parameters: 95°C for 10 min; 40 cycles of 95°C for 30 s; 62°C for 30 s; 72°C for 30 s; read plate, 76°C for 2 s; read plate, 78°C for 2 s; read plate, 80°C for 2 s; read plate, 81°C for 2 s; then read plate 72°C for 1 min. PCR primers were as follows: 5’-TGACTTTCACGACACGTTTCTGGC-3’ for *prpD* forward; 5’-TGTGGATCTCATAGGCGGTACCA-3’ for *prpD* reverse; 5’-ATGGCTGGACATCTACCA GGTGT-3’ for *prpC* forward; and 5’-TGTCGAATCCCATCAGGTAGTACG-3’ for *prpC* reverse. We calculated relative abundance of message by comparing all amounts for each gene relative to a standard curve from the H37Rv strain in cholesterol for the corresponding gene. The quantity of cDNA in each sample was normalized to the *sigA* mRNA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at doi:10.1016/j.chembiol.2011.12.016.

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