Propionate metabolism in *Mycobacterium tuberculosis*: Characterization of the vitamin B$_{12}$-dependent methylmalonyl pathway

Suzana Anna Savvi

A dissertation submitted to the Faculty of Science
University of the Witwatersrand, Johannesburg,
for the degree of Doctor of Philosophy
December 2008.
When you are inspired by some great purpose, some extraordinary project, all your thoughts break their bonds: Your mind transcends limitations, your consciousness expands in every direction, and you find yourself in a new, great, and wonderful world. Dormant forces, faculties and talents become alive, and you discover yourself to be a greater person by far than you ever dreamed yourself to be.

Patanjali
DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination at any other university.

10 December 2008

__________________________________________  ________________________
Suzana Anna Savvi                                      Date
Propionyl-CoA is a three-carbon (C₃) short-chain fatty acid (SCFA) derivative of branched-chain amino acids, branched- and odd-chain fatty acids and cholesterol. Degradation of propionyl-CoA-generating carbon sources during infection (Pandey and Sassetti, 2008) requires the concomitant ability to oxidise this metabolite as a carbon and energy source, so as to avoid its cytotoxic effects if accumulated. The methylcitrate cycle in *Mycobacterium tuberculosis* (MTB) has been characterized and is essential for propionate oxidation *in vitro*, although dispensable for growth and persistence in mice (Muñoz-Elias *et al.*, 2006). This study reveals that MTB possesses an alternative pathway for propionate metabolism, the vitamin B₁₂-dependent methylmalonyl pathway. Specifically, we demonstrate the ability of MTB to utilise propionyl-CoA-generating carbon sources in the absence of the methylcitrate cycle, provided that vitamin B₁₂ is supplied exogenously. This ability is shown to be dependent on methylmalonyl-CoA mutase (MCM; MutAB), which requires the adenosylcobalamin derivative of vitamin B₁₂ for activity. The inability of MTB to synthesise vitamin B₁₂ (Warner *et al.*, 2007) is consistent with the essentiality of the methylcitrate cycle for growth on propionate (Muñoz-Elias *et al.*, 2006). The demonstrated functionality of the methylmalonyl pathway offers an explanation for the dispensability of the methylcitrate cycle for survival of the mycobacterium *in vivo* where access to vitamin B₁₂ may be unrestricted.

Gene expression analysis was used to interpret flux through the two pathways on propionate (C₃) and valerate (C₅) odd-chain fatty acids. In the presence of a functional methylmalonyl pathway, expression of methylcitrate dehydratase (MCD) and methylcitrate lyase (MCL) was reduced. Consistent with reduced levels of bifunctional isocitrate lyase (ICL)1/ MCL in MTB (Gould *et al.*, 2006; Muñoz-Elias *et al.*, 2006), growth on propionate and valerate was shown to by-pass the requirement for carbon anaplerosis by the glyoxylate cycle when propionyl-CoA was converted to the tricarboxylic acid cycle (TCA) intermediate, succinyl-CoA, through
the methylmalonyl pathway. The potential of an autonomous methylmalonyl pathway in MTB is demonstrated which underscores the importance of vitamin B$_{12}$ in MTB physiology.

Alternately, MTB deficient for the methylcitrate cycle was able to grow on heptadecanoate (C$_{17}$) without vitamin B$_{12}$ supplementation. In the absence of either propionate oxidizing pathway, derivative propionyl-CoA may be used as a key precursor for the biosynthesis of several cell wall virulence lipids (Jain et al., 2007).
ACKNOWLEDGMENTS

I am grateful to the Medical Research Council of South Africa, the National Research Foundation, the Howard Hughes Medical Institute (International Research Scholars grant to V.M.), the NHLS Research Trust, the National Health Laboratory Service and University of the Witwatersrand for financial support, and for contributing to local and international travel expenses.

I wish to thank John McKinney for a rewarding visit to his laboratory at Rockefeller University and for the dynamic collaboration with himself, Anna Upton and Manisha Lotlikar. Your kind generosity and advice has been invaluable in shaping this research.

I am indebted to all my colleagues at the Molecular Mycobacteriology Research Unit for collectively creating a working environment conducive to great scientific discovery. Bavesh, your impressions of wisdom are indelible. You are a dear friend. Bhavna Ghordan, thank you for your patient training in the BSL3 facility without which this work would not have been possible. I am immeasurably grateful to Diane Kuhnert for facilitating the biochemistry aspect of this study. And to Garth Abrahams who never ceased to assist.

Digby Warner, you have nurtured our scientific ideas with unmatched analytical dexterity and softened disappointment with elegant humor. I cannot express enough gratitude for your encouragement and assistance in every facet of this work.

I am most indebted to Stephanie Dawes who has supervised this work with cool reasoning and unfailing enthusiasm. You have showed me new ways of seeing things, redefined my mental perception so that today I may emerge a scientist. God surely had a hand in placing me under the guidance of a rare and exceptional prodigy.

It has been an enormous privilege to work under the auspices of Valerie Mizrahi, both as my supervisor and mentor. You gave me an opportunity which altered the course of my life, thank you. Through these years I have so often been in awe of your infallible reasoning and remarkable scientific deduction, an endless source of inspiration which I will miss.
To my Mother, Father and Sister for their unwavering support and commitment to the completion of this chapter in life. I can only hope that in this accomplishment lies measurable reward for the sacrifices made. Thank you. And to my children who have felt the sorrows and joys of this experience with me, know this is dedicated to you.
PUBLICATIONS FROM THIS THESIS


TABLE OF CONTENTS

DECLARATION.................................................................................................................. iii

ABSTRACT ......................................................................................................................... iv

ACKNOWLEDGMENTS..................................................................................................... vi

PUBLICATIONS FROM THIS THESIS............................................................................... viii

TABLE OF CONTENTS .................................................................................................... ix

LIST OF FIGURES ............................................................................................................ xi

LIST OF TABLES ............................................................................................................... xii

1 INTRODUCTION .......................................................................................................... 2
  1.1 Resurgence of tuberculosis ...................................................................................... 2
    1.1.1 Disease pathology ............................................................................................ 3
    1.1.2 TB chemotherapy .......................................................................................... 5
  1.2 Persistence of MTB and its role in defining antitubercular therapy ......................... 7
  1.3 Intraphagosomal survival of MTB ........................................................................... 9
    1.3.1 The phagosome environment ......................................................................... 9
    1.3.2 Metabolic adaptations of MTB within the phagosome .................................. 11
    1.3.3 The nutrient status within phagosomes ....................................................... 13
    1.3.4 Cell envelope shedding ................................................................................. 14
  1.4 Carbon metabolism in MTB .................................................................................. 16
    1.4.1 Interpreting carbon usage in vivo through metabolic pathway assessment .. 16
    1.4.2 Regulation of carbon metabolism in MTB ..................................................... 21
    1.4.3 The sources and types of nutrients in vivo ................................................... 23
  1.5 Propionate metabolism ......................................................................................... 26
    1.5.1 Characterised pathways for propionyl-CoA catabolism .................................. 28
    1.5.2 The adenosylcobalamin-dependent methylmalonyl-CoA mutase reaction .... 29
    1.5.3 Anaplerosis by the methylmalonyl pathway ............................................... 31
  1.6 The anabolic role of MCM in polyketide synthesis ................................................. 34

2 AIMS and OBJECTIVES ............................................................................................... 37

3 MATERIALS and METHODS ....................................................................................... 38
  3.1 Bacterial strains and culture conditions ................................................................. 38
  3.2 Construction and complementation of mutant strains of MTB ............................. 38
  3.3 Heterologous over-production of a His-tagged recombinant form of Rv1998c in E. coli 40
  3.4 Tetracycline-inducible expression of Rv1998c in MTB ........................................ 41
  3.5 Preparation of MTB cell-free extracts ................................................................... 42
  3.6 2- Methylcitrate lyase assays ................................................................................. 43
  3.7 Gene expression analysis by real-time quantitative reverse transcription-PCR (qRT-PCR) 43
4 RESULTS .................................................................46
 4.1 Pathways for propionate metabolism in MTB deduced from bioinformatic analysis ........46
 4.2 Constituents of the methylmalonyl pathway .................................................................48
 4.3 Vitamin B\textsubscript{12} supplementation enables a functional methylmalonyl pathway ....52
 4.4 Constituents of the methylcitrate cycle .................................................................55
 4.4.1 Identification of Rv1998c as a putative MCL ..................................................56
 4.4.2 Analysis of the MCL function of Rv1998c ................................................56
 4.4.2.1 Over-expression of Rv1998c in \textit{E. coli} .........................................................60
 4.4.2.2 Induction of Rv1998c expression in MTB and MCL assay of MTB cell extracts ....62
 4.4.3 Deletion of Rv1998c in the \textit{Δicl1} mutant of MTB Erdman ..................................65
 4.5 Growth of MTB H37Rv on propionate is attributable to the methylcitrate cycle ....66
 4.6 Vitamin B\textsubscript{12} supplementation enables growth of a propD\textsuperscript{DC} mutant ....67
 4.7 Determining propionyl-CoA partitioning through the methylcitrate cycle and methylmalonyl-CoA pathway by gene expression analysis .................................................................70
 4.8 Growth of MTB on propionate in the absence of the glyoxylate cycle ......................73
 4.9 Emergence of suppressor mutants in response to metabolite stress .......................77
 4.10 Growth on C\textsubscript{5} and C\textsubscript{17} carboxylic acids ........................................79
 4.10.1 Growth on valerate ....................................................79
 4.10.2 Comparative analyses of prop\textsubscript{D}, icl and mutB expression on different carbon sources 81
 4.10.3 Growth on heptadecanoate ...................................................84
 4.11 Growth of MTB on acetate with vitamin B\textsubscript{12} ............................................86

5 DISCUSSION ..........................................................88
 5.1 The MTB gene complement for propionate oxidation ...........................................88
 5.2 MCM is not essential for propionate metabolism in MTB .........................................89
 5.3 Vitamin B\textsubscript{12} supplementation facilitates operation of the methylmalonyl pathway ....89
 5.4 The unresolved function of Rv1998c in MTB .............................................................90
 5.5 Conditional essentiality of \textit{ΔpropDC} for growth on propionate .........................93
 5.5.1 A functional methylmalonyl pathway in MTB .................................................94
 5.6 Transcriptional analysis of prop\textsubscript{D}, icl and mutB ...........................................97
 5.7 Bypassing the requirement for the glyoxylate cycle for growth on propionate by the methylmalonyl pathway .................................................................99
 5.8 Genotypic adaptation of \textit{Δicl1} to growth on B\textsubscript{12}supplemented propionate ......101
 5.9 Growth on valerate as a carbon source ..............................................................103
 5.9.1 Anaplerotic role for the methylmalonyl pathway on valerate .................................104
 5.10 Growth on heptadecanoate as a carbon source .....................................................106
 5.11 Propionyl-CoA incorporation into virulence lipids relieves toxicity on valerate ....110
 5.11.1 Maintaining methylmalonyl-CoA homeostasis in MTB ....................................112
 5.12 A role for MCM in acetate assimilation .............................................................113

6 CONCLUDING REMARKS ..................................................................................115

7 APPENDICES ..............................................................................................117
7.1 Appendix 1: List of Abbreviations ....................................................................................117
7.2 Appendix 2: Culture Media .............................................................................................120
8 References ........................................................................................................................122

LIST OF FIGURES

Fig. 1 Anaplerotic pathways in MTB ............................................................................................21
Fig. 2 Predicted pathways of propionate metabolism in MTB ..................................................48
Fig. 3 Phylogenetic analysis of β subunits of ACCase complexes found in actinomycetes ..........49
Fig. 4 Phylogram of MutB orthologues .......................................................................................50
Fig. 5 Construction and characterisation of the ΔmutAB mutant of MTB H37Rv .......................54
Fig. 6 Amino acid alignment of representatives of the ICL/PEPM superfamily .........................57
Fig. 7 Model of Rv1998c fitted to PrpB of E. coli .......................................................................59
Fig. 8 Expression of recombinant Rv1998c in E. coli .................................................................61
Fig. 9 SDS-PAGE of soluble cell extract after affinity chromatography on Ni-Sepharose column ..........................62
Fig. 10 Schematic of construct used for Tet-regulated expression of Rv1998c in MTB ..................64
Fig. 11 The methylcitrate cycle is required for growth of H37Rv on propionate .........................67
Fig. 12 Growth on propionate supplemented with 10 µg/ml vitamin B12 .......................................69
Fig. 13 Vitamin B12 supplementation enables mutAB-dependent growth of a prpDC-deficient mutant of H37Rv on propionate ........................................................................69
Fig. 14 Effect of vitamin B12 (cyanocobalamin) concentration on growth of the ΔprpDC mutant on propionate 70
Fig. 15 Quantitative expression analysis of methylcitrate cycle and methylmalonyl pathway genes in MTB ......73
Fig. 16 The methylmalonyl pathway enables bypass of the glyoxylate cycle allowing growth of Δicl1 on propionate supplemented with vitamin B12 ..............................................................75
Fig. 17 The methylmalonyl pathway enables bypass of the glyoxylate cycle during growth of MTB on propionate ........................................................................................................76
Fig. 18 Vitamin B12-dependent growth in propionate of a suppressor mutant after serial passage in 7H9 ..........78
Fig. 19 Growth of MTB on valerate as a propionyl-CoA precursor .............................................80
Fig. 20 Expression of prpD, icl and mutB genes of H37Rv cultured on propionate or valerate in the presence of vitamin B12 ........................................................................................................81
Fig. 21 Anaplerotic role for MCM revealed by growth of MTB on valerate with 3NP and vitamin B12 supplementation ......................................................................................................................82
Fig. 22 The operation of an autonomous methylmalonyl pathway bypasses the requirement for the glyoxylate cycle during growth of MTB on valerate ..........................................................83
Fig. 23 Growth of MTB on heptadecanoate (C17) ......................................................................85
Fig. 24 Growth comparison on valerate between two variable PDIM-producing strains of H37Rv ................86
Fig. 25: Citramalate cycle adapted from Ivanovsky et al (2002) and Textor et al (1997) .................93
Fig. 26 Proposed model detailing the variable anaplerotic contribution of the methylmalonyl pathway relative to the glyoxylate cycle on odd-chain fatty acids........................................................109
LIST OF SUPPLEMENTARY FIGURES

Fig. S. 1 Vector Maps .................................................................................................................. 154
Fig. S. 2 Southern blots .............................................................................................................. 155

LIST OF TABLES

Table 1 Conditional expression of Rv1998c in MTB .................................................................. 64
Table 2. Growth on MTB on acetate supplemented with 3NP and vitamin B12 ................................ 87

LIST OF SUPPLEMENTARY TABLES

Table S. 1 Strains and plasmids used in this study ..................................................................... 156
Table S. 2 Oligonucleotides used in this study ......................................................................... 158
1 INTRODUCTION

1.1 Resurgence of tuberculosis

Tuberculosis (TB) is a chronic disease caused by the etiological agent, *Mycobacterium tuberculosis* (MTB). About one third of the world’s population – an estimated 1.8 billion people – is believed to be latently infected with MTB (Dye et al., 1999). The highest incidence rates of TB are in sub-Saharan Africa and are associated with the high prevalence of HIV infection (Corbett *et al.*, 2003). The annual risk of TB increases dramatically in immunocompromised patients as a result of immune system perturbations by HIV (Wood *et al.*, 2000), consistent with coinfection rates of >50% in southern African countries (Dye, 2006). The synergy between MTB and HIV (Collins *et al.*, 2002b) makes for a lethal combination which, together with the world-wide emergence of drug-resistant strains of MTB introduced by the selective pressure imposed by more than 30 years of short-course chemotherapy (Anon, 1981, 1982), and the parallel demographic evolution of expanding pathogen and patient populations (Wirth *et al.*, 2008), has elevated the magnitude of concern about the TB epidemic. Measures for controlling this disease need to be significantly improved if the estimated figures for between 2000 and 2020, where 35 million people are predicted to die from TB, are to be reduced (www.who.int/tb/strategy/en/).

Immunization as an efficient and cost-effective strategy against TB is part of the World Health Organization’s (WHO) expanded vaccination program. Since the 1920s, an attenuated derivative of MTB, bacillus Calmette–Guérin (BCG) (Behr *et al.*, 1999), has been widely used as a prophylactic TB vaccine because of its proven efficacy at preventing TB meningitis and miliary TB in children (Cohn, 1997). However variable protective efficacy against pulmonary disease is displayed in adults and may stem from high diversity among BCG strains (Brosch *et al.*, 2007). This translates into BCG having little impact on the global prevalence and epidemiology of TB.
1.1.1 Disease pathology

The inhalation of mycobacterial-containing droplets and subsequent engulfment by alveolar macrophages and dendritic cells in the airways stimulates a robust proinflammatory response through the activity of Toll-like receptor agonists abundant on the surface of the bacteria. Interaction with these antigen presenting cells (APC) results in the production of inflammatory cytokines, including tumor necrosis factor (TNF) and interleukin (IL)-12 (Henderson et al., 1997; Hickman et al., 2002). This drives the recruitment of various immune cells types to the infection site, which collectively, are responsible for mediating containment of infection through the formation of the ‘tubercle’ or granuloma after which the disease is named (Bishai, 2000). Specifically, activated dendritic cells traffic to the secondary lymphoid organs and prime naïve T cells to initiate the adaptive immune response (Banchereau and Steinman, 1998). A strong T cell response is initiated, dominated by effector CD4+ T cells of the Th1 type (Flynn and Chan, 2001; Kaufmann et al., 2005). These secrete IFN-γ and TNF-α, which in turn induces antimycobacterial mechanisms in macrophages, including the production of reactive oxygen and nitrogen intermediates (Ding et al., 1988; Flynn and Chan, 2001). The markedly enhanced incidence of TB in individuals that are coinfected with HIV indicates that the CD4+ T cell defense is clearly a crucial element of the cellular immune response, and correlates with reduced granuloma-forming capacity and dissemination throughout the host (Kaufmann and McMichael, 2005).

The mature-phase granulomatous lesion has a central necrotic core surrounded by concentric layers of macrophages, epithelioid cells, multinucleated Langhans giant cells and lymphocytes enclosed within a fibrotic capsule with surrounding peripheral lymphoid follicular structures orchestrating the local immune response (Ulrichs et al., 2004; Ulrichs and Kaufmann, 2006). Successful containment of the pathogen results in latent infection. Various models have documented the distinct spatio-temporal dynamics of infiltrating leukocytes during the process of granuloma formation (Lin et al., 2006a; Segovia-Juarez et al., 2004; Tsai et al., 2006). Notably, mice do not develop such highly organised structures to contain infection but rather develop chronic multibacillary infection. As a consequence, mice may have less tissue
damage and give faster colony forming unit (CFU) clearance during TB drug therapy (Jain et al., 2008). Therefore the granuloma is considered to serve as an environmental niche in which mycobacteria can survive, effectively screened from active immune surveillance (Grosset, 2003). Bacteria persist under the low oxygen tension (Sherman et al., 2001; Via et al., 2008; Voskuil et al., 2003) and lipophilic environment within the granuloma (Honer Zu Bentrup et al., 1999; McKinney et al., 2000; Muñoz-Elias and McKinney, 2005) by adjusting their metabolism, allowing for transition to dormant-like nonreplicative state.

Only between 5-10% of individuals who become infected with MTB subsequently develop clinical disease (Bishai, 2000; Bloom and Murray, 1992). In the other 90% the interplay between adaptive and innate immunity serves to control the infection with no obvious clinical symptoms. These individuals are in a state of latent TB infection (LTBI) which may persist for many years without ever developing into active disease. Latent infection with MTB is estimated to affect at least 2 billion people worldwide (Dye et al., 1999). Several effector molecules released by MTB at the infection site promote the survival of viable bacilli during latent infection (Russell et al., 2002; Russell, 2007). For instance, interaction of a mycobacterial mannose-containing cell wall ligand with DC-specific C-type lectin, DC-SIGN, induces the immunosuppressive cytokine IL-10 (Geijtenbeek et al., 2003; Tailleux et al., 2005), contributing to the survival of MTB during latent infection. Such mechanisms actively deployed by the bacilli to achieve subversion of host immune responsiveness, combined with host mechanism to prevent exacerbated immunopathology during chronic infection, where regulatory T cells (T\text{reg}) are responsible for down-modulating the immune responses (Kursar et al., 2007), result in low level persistent infection rather than sterile eradication of this pathogen. Therefore, latently infected individuals represent a large reservoir of MTB.

Reactivation occurs when the immunological balance between pathogen and host is disturbed as a consequence of several possible host and environmental factors such as TB/HIV co-infection (Corbett et al., 2003), the use of immunosuppressive agents (Jacobs et al., 2007) possible host genetic factors (Caws et al., 2008) and other factors such as malnutrition, ageing and stress. Peptidoglycan hydrolases otherwise known as resuscitation-promoting factors have been implicated in reactivation from chronic TB (Biketov et al., 2007; Russell-Goldman et al.,
The morphological heterogeneity that may exist in human granulomas demonstrates the continuum between active and non-progressive lesions (Lin et al., 2006a). A progressive lesion is characterised by caseous necrosis that precedes erosion into the bronchus and promotes transmission (Reichler et al., 2002; Rodrigo et al., 1997). By coughing, a patient with tuberculous lung cavities aerosolises and disseminates the bacilli. During this active stage of disease, TB is highly contagious (Escombe et al., 2008), and can transmit to between 10 and 15 people a year from a single infected person (Riley et al., 1962; Riley et al., 1995; Sultan et al., 1960). Given the high numbers of infected individuals, reducing the incidence of TB and the rate of MTB transmission presents enormous challenges.

1.1.2 TB chemotherapy

Since the risk of disease is highly dependent on the immune status of the host, susceptibility to reactivation increases dramatically to 5-15% annually in persons coinfected with HIV (Corbett et al., 2003; Raviglione et al., 1995). Considering that both pulmonary and disseminated forms of disease develop in these individuals, accounting for 25% of AIDS deaths worldwide, the WHO and its international partners have formed the TB/HIV Working Group (http://www.stoptb.org/wg/tb_hiv/) in response to the challenges presented by the entwined epidemics of TB and HIV. This group develops global policy on the control of HIV-related TB, and tackles issues such as ‘immune reconstitution inflammatory syndrome’ (IRIS), a poorly understood phenomenon that affects those taking drugs for both TB and HIV. In the case of IRIS, initiating antiretroviral therapy alone seems to severely aggravate TB in dually infected patients, which further compounds the problems associated with co-infection. The Stop TB Strategy, launched in 2006 by the WHO, has integrated these challenges as one of the six components of its enhanced, core-based directly observed treatment short-course (DOTS) programme (http://www.who.int/tb/strategy/en/index.html).

The DOTS-based service was launched in 1995 to address the operational problem that effective TB chemotherapy requires a lengthy 6-9-month treatment with a combination of the first line drugs, rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutanol (EMB) (el-Sadr et al., 1998). Despite the global implementation of DOTS-based TB control
programmes, the limited effectiveness of current therapy is evidenced by the emergence and spread of multi (MDR) and extensively drug-resistant (XDR) strains of MTB, where MDR is defined as resistance to the first-line drugs, INH and RIF. This development, which is severely compromising TB control efforts, has been driven by the lack of compliance with the lengthy and complicated treatment regimen and the use of suboptimal drug concentrations. When available, second-line drugs, which include the fluoroquinolones (ciprofloxacin, ofloxacin, moxifloxacin, gatifloxacin and levofloxacin), the injectable drugs (amikacin, kanamycin and capreomycin) and others, such as ethionamide, D-cycloserine and p-aminosalicylic acid, are administered to patients who fail to respond to first-line therapy, albeit often under poorly supervised DOTS programmes. Second-line drug treatment is fraught with drawbacks such as adverse side effects and prolonged duration of therapy (up to 2 years), further limiting patient adherence and fuelling the rise of XDR-TB, which is due to infection with strains that are resistant to any fluoroquinolone, and at least one of three injectable second-line drugs (capreomycin, kanamycin and amikacin), in addition to INH and RIF (http://www.euro.who.int/Document/Mediacentre/fs0107e.pdf). Propagation of these highly resistant strains by nosocomial transmission in communities with high HIV prevalence and poor TB control practices contributed to the recent and highly publicised outbreak of XDR-TB at Tugela Ferry in the KwaZulu-Natal province of South Africa (Gandhi et al., 2006). In this study, 53 cases of XDR-TB were identified from 221 MDR-TB cases identified in the area in 2005. Moreover, XDR strains of MTB were found to be transmitted to HIV co-infected patients and were associated with high mortality (Gandhi et al., 2006; Singh et al., 2007b). A subsequent molecular epidemiological analysis of this outbreak revealed that the emergence and clonal spread of XDR-TB that occurred in this region over this past decade was attributable to strains belonging to the F15/LAM4/KZN strain family, with the lack of routine drug susceptibility testing as an integral part of DOTS in an ever expanding HIV positive society being identified as the major factor underlying this unfortunate outbreak (Pillay and Sturm, 2007).

Clearly new treatment-shortening therapies that also are effective against MDR and XDR strains of MTB are urgently required. Mathematical modeling of the impact of treatment
duration on TB dynamics have suggested that a 2-3 fold increase in rates of decline could be achieved should treatment duration be reduced to 2 months (Salomon *et al*., 2006), emphasizing the importance of drug discovery research that is aimed specifically at shortening the duration of therapy. A number of promising lead compounds and drug candidates are in various stages of clinical development to direct TB therapy closer towards achieving these goals. A novel drug class for TB treatment is exemplified by the diarylquinoline, TMC207, which was originally identified by empiric whole cell screening against *M. smegmatis* (MSM), and which has been demonstrated to inhibit the mycobacterial ATP synthase (Andries *et al*., 2005; Koul *et al*., 2007). Another novel class of compounds in the TB drug development pipeline is the nitroimidazoles, which includes PA-824 (Manjunatha *et al*., 2006) and OPC-67863 (Matsumoto *et al*., 2006). Both compounds are prodrugs whose nitroreductive activation is a prerequisite for bactericidal activity but whose precise mechanism of action has not been resolved fully (Manjunatha *et al*., 2006; Matsumoto *et al*., 2006; Singh *et al*., 2008).

Although these drugs may make significant contributions to future TB therapy, further knowledge of the mechanisms of persistence and proliferation of MTB are necessary in order to uncover novel essential metabolic targets against which drugs having improved efficacy could be designed, thereby simplifying and shortening TB chemotherapy. Specifically, discovering and evaluating new compounds depends on the metabolic state of the organism, which directly impacts on drug susceptibility as exemplified by resistance to fosfomycin in *Listeria monocytogenes*, largely an *in vitro* phenotype as expression of a virulence factor during mouse infection is responsible for *in vivo* susceptibility (Scortti *et al*., 2006).

1.2 Persistence of MTB and its role in defining antitubercular therapy

A testimony to the resilience of MTB is highlighted by the ability of this pathogen to survive for extended periods in the asymptomatic host in a state of clinical latency from which it can reactivate and to persist, possibly in a quiescent, physiological state of non-replication during active disease in the face of administered chemotherapy. The issue of how the viability of MTB can be maintained in the absence of growth is the subject of intense interest and debate (Boshoff and Barry, 2005) although there is little information available on the metabolic status
of the organism in a persistence state. When bacteria were cultivated in microfluidic chambers and their behavior recorded using time–lapse video microscopy, *Escherichia coli* persisters were identified as a pre-existing subpopulation in an arrested growth state (Balaban *et al.*, 2004). Studies have suggested that despite the stability in mycobacterial colonization during the chronic stage of infection in the mouse model (Muñoz-Elias *et al.*, 2005), the bacilli remain metabolically active (Talaat *et al.*, 2007). The slowing down or arrest of bacterial replication during the chronic state of infection and in persister organisms is thought to be at least partly responsible for increased phenotypic drug tolerance (Paramasivan *et al.*, 2005). New drugs will need to overcome this refractoriness, although their development is contingent upon greater knowledge of persistence during active disease or latent infection.

To facilitate research in this area, a number of *in vitro* models of latency and persistence have been established. A model based on the gradual depletion of oxygen which causes MTB to shift from active replication into a state of non-replicating persistence that may resemble the physiological state of quiescent tubercle bacilli *in vivo*, was used to explore the lethal effect of metronidazole against dormant bacilli under anaerobic conditions (Wayne, 1976; Wayne and Sramek, 1994; Wayne and Hayes, 1996). Similarly, the related nitroimidazole antibiotic, PA-824, possessed activity against static non-replicating MTB in this anaerobic culture model (Stover *et al.*, 2000). Stationary phase cultures have similarly been adopted to model non-growing persister MTB tolerant to antibiotics (Hu *et al.*, 2000; Hu *et al.*, 2003). Such models have been used in whole cell screens of compound libraries and identified candidates with significant activity against non-growing bacilli (Bryk *et al.*, 2008; Byrne *et al.*, 2007). For example, rhodanines are the most recent class of compounds identified able to kill nonreplicating bacteria (Bryk *et al.*, 2008). Further clinical development is contingent on suitable pharmaceutical properties and drug-drug compatibility (Balganesh *et al.*, 2008) given which, prophylaxis in individuals with latent MTB infection is proposed (Bryk *et al.*, 2008).

To validate persistence drug targets, powerful new genetic systems for conditional gene silencing have been developed and are being applied (Blokpoel *et al.*, 2005; Ehrt *et al.*, 2005). Application of this approach to the MTB 20S proteasome core revealed an unexpected requirement of the proteasome for persistence of MTB during the chronic phase of infection in mice (Gandotra *et al.*, 2007). An improved understanding of the mechanisms governing
persistence, in combination with conditional gene silencing, will increase the number of validated persistence targets for drug discovery, which will ultimately shorten and simplify therapy.

1.3 Intraphagosomal survival of MTB

1.3.1 The phagosome environment

Deep within the pulmonary alveoli, inhaled mycobacteria bind and enter resident alveolar macrophages, dendritic cells and monocytes by binding to multiple receptors. These myeloid cells have a significant role in innate host resistance to infection representing the first line of defense. Phagocytosis by the predominant complement receptor type 3 (CR3) (Schlesinger et al., 1990) in a cholesterol-dependent manner (Gatfield and Pieters, 2000; Peyron et al., 2000) enables entry into macrophages. These membrane-bound organelles termed phagosomes become privileged replicative niches of MTB by interference of phagolysosomal biogenesis (Goren et al., 1976; Vergne et al., 2005). Through the manipulation of host signal transduction pathways, MTB maintains extensive communication with early endosomal traffic (Vergne et al., 2004) promoting access to nutrients for survival and growth (Russell et al., 1996; Sturgill-Koszycki et al., 1996; Vergne et al., 2004). The capacity of mycobacteria to block phagosome-lysosome fusion is influenced by several virulence factors (Chua et al., 2004; Russell et al., 2002). Some recognised mechanisms by which MTB modulates host cell trafficking pathways thereby inhibiting phagosomal maturation include the active recruitment and retention of coronin-1 on mycobacterial phagosomes (Ferrari et al., 1999; Jayachandran et al., 2007), hydrolysis of the membrane trafficking regulatory lipid essential for phagosomal acquisition of lysosomal constituents, phosphatidylinositol 3-phosphate (PI3P) by MTB secreted lipid phosphotase (Fratti et al., 2001; Vergne et al., 2005) and MTB eukaryotic-like protein kinase G (PknG)-directed signal transduction (Walburger et al., 2004). Advances in identifying host factors required for mycobacterial survival in the phagosome are lending further insights into how mycobacteria manipulate the host cell (Kuijl et al., 2007; Philips et al., 2008). The consequence of preventing phagosome-lysosome fusion is a compartment with
limited phagosomal acidification and hydrolytic capacity (Mwandumba et al., 2004; Russell et al., 2002).

The macrophage’s antimicrobial defense mechanisms extend beyond fusion of phagosomes with lysosomes and include other potent mechanisms such as nutrient limitation (Muñoz-Elias and McKinney, 2006), acidic pH (Deretic and Fratti, 1999), the synergistic action of fatty acids and host immune effectors such as reactive oxygen and nitrogen intermediates (Akaki et al., 2000; Chan et al., 1992; Hemsworth and Kochan, 1978; Saito and Tomioka, 1988) and the recently discovered host-derived inhibitory factor, carbon monoxide (CO) (Kumar et al., 2008; Shiloh et al., 2008). The adaptive metabolic strategies adopted to survive in the hostile intraphagosomal environment have been probed using in vitro models (Betts et al., 2002; Dahl et al., 2003; Fisher et al., 2002; Hampshire et al., 2004; Manganelli et al., 2001; Voskuil et al., 2003; Wayne and Hayes, 1996), showing significant overlap in RNA expression between infected macrophage (Fontan et al., 2008; Schnappinger et al., 2003), murine and human lung tissue (Fenhalls et al., 2002; Shi et al., 2005; Talaat et al., 2007; Timm et al., 2003). A common feature that has emerged in response to the various intraphagosomal stresses is the induction of the DosR regulon (Kendall et al., 2004; Schnappinger et al., 2003), a set of approximately 50 genes induced in response to hypoxia, CO and nitric oxide (NO) (Kumar et al., 2008; Park et al., 2003; Sherman et al., 2001; Shiloh et al., 2008; Voskuil et al., 2003), which is controlled by a two-component regulator system comprising the sensor kinases DosS and DosT and the cognate response regulator, DosR (Kumar et al., 2007; Roberts et al., 2004). The DosR regulon includes genes of anaerobic respiration and stabilization of vital cellular components, and together with the Enduring Hypoxic Response (EHR) is thought to play an important role in MTB adopting a quiescent physiological state (Ohno et al., 2003; Roberts et al., 2004; Rustad et al., 2008; Voskuil et al., 2003). The generation of NO by IFN-γ-activated macrophages induces an environment inhibitory of aerobic respiration (Brown, 2001). Hypoxia-mediated bacteriostasis in vitro (Voskuil et al., 2003; Wayne and Hayes, 1996) shares similarities to NO induced transition of MTB from active growth to nonreplicating persistence in mice (Karakousis et al., 2004; Shi et al., 2005), supporting the notion that NO production (together with granuloma formation) by the immune system limits aerobic respiration of MTB and impairs growth.
1.3.2 Metabolic adaptations of MTB within the phagosome

The overall theme that has emerged from such studies is that MTB undergoes a switch to microaerophilic and lipid metabolism inside the phagosome. The down-regulation of genes encoding subunits of NADH dehydrogenase (NDH)-I, the menaquinol-cytochrome c complex, and ATP synthase collectively reflect the reduced need for energy generation as a function of adaptation to a low-growth state inside the macrophage (Beste et al., 2007; Schnappinger et al., 2003). The concomitant up-regulation of the type II NADH dehydrogenase, NDH-2, probably signals the increased need for regenerating reducing equivalents for β-oxidation (Schnappinger et al., 2003). In other organisms, NDH-2 acts as the electron donor for the electron transport chain (ETC) under anaerobic conditions (Boshoff and Barry, 2005; Rao et al., 2008; Shi et al., 2005). No ATP is generated by substrate level phosphorylation through β-oxidation (Boshoff and Barry, 2005; Schnappinger et al., 2003). In this instance, ATP synthesis by ATP synthase may be essential for viability, as demonstrated in carbon-limited cultures (Koul et al., 2008; Rao et al., 2008). ATP synthase activity is contingent on the NDH-2-dependent proton motive force (PMF) (Rao et al., 2008), using fumurate reductase and/or succinate dehydrogenase with fumurate or nitrate reductase with nitrate as the electron acceptors (Boshoff and Barry, 2005; Rao et al., 2008). The NDH-2 therefore has bimodal action in (i) the regeneration of reducing equivalents; and (ii) maintaining the PMF required for ATP synthesis. Limiting terminal electron acceptors, fumurate or nitrate, in culture is expected to phenocopy inhibition of NDH-2 by similarly restricting the recycling of reducing equivalents and disrupting the PMF and therefore, ATP synthesis. However other enzymes such as isocitrate lyase (ICL) with glycine dehydrogenase may contribute to the maintenance of NAD⁺ pools when cultures are grown on fatty acid carbon sources (Wayne and Lin, 1982). The redox balance may be maintained by also sinking cellular reducing equivalents into triacylglycerol synthesis (Reed et al., 2007).

Interestingly, the genes predicted to be involved in NAD⁺ regeneration are not actively induced or repressed during the EHR suggesting that in the absence of respiration, other
pathways operate during hypoxia to generate energy (Boshoff and Barry, 2005; Rustad et al., 2008). This may be reflected by the survival of bacilli that are recalcitrant to treatment with the ATP synthase inhibitor, R207910, in the hypoxic granulomas that are formed in guinea pigs (Lenaerts et al., 2007). In MSM, maintenance of an energised membrane is not dependent on the activity of ATP synthase (Koul et al., 2008), suggesting that the sole function of ATP synthase is in ATP synthesis. Conceivably, substrate level phosphorylation may function instead to meet the cellular demand for ATP. This is exemplified in other bacteria where loss of oxidative phosphorylation is compensated by increased flow through the glycolytic pathways and TCA cycle to enhance substrate level phosphorylation on fermentable carbon sources (Jensen and Michelsen, 1992; Santana et al., 1994). However, the essentiality of ATP synthase in MSM, even on fermentable substrates suggests that substrate level phosphorylation is unable to meet the cellular demand for ATP (Tran and Cook, 2005). In MTB, ATP synthase may be essential (Sassetti et al., 2003) under the conditions tested either due to (i) the insufficient generation of ATP by substrate level phosphorylation, or (ii) the obligatory requirement for the oxidation of NADH by providing a sink for translocated protons. In the event that the latter is not a prerequisite, it has been proposed that substrate level phosphorylation may be enhanced by activating the reversible succinyl-CoA→methylmalonyl-CoA→propionyl-CoA→propionate pathway (methylmalonyl pathway), (Kana et al., 2009) which could generate 2 ATP molecules per succinyl-CoA, provided the coenzyme $B_{12}$ requirements of the methylmalonyl-CoA mutase (MCM) are met (discussed in section 1.5.1). In MSM, no growth on succinate was observed by a ATP synthase mutant (Tran and Cook, 2005), although the relevance of the methylmalonyl pathway for viability of this mutant strain was not assessed.

The propensity of MTB to slow down its metabolism (Beste et al., 2007) may contribute to the tolerance displayed by the bacterium to adverse conditions although the ability of MTB to survive within macrophages is more than merely a passive retreat into dormancy. MTB remains metabolically active (Lee et al., 2008; Talaat et al., 2007) displaying active defense (Darwin et al., 2003; Davis et al., 2007; Vandal et al., 2008) and physical properties (Chan et al., 1991) against macrophage effector functions, enabling the maintenance of acute and persistent infections (Zahrt and Deretic, 2002).
1.3.3 The nutrient status within phagosomes

The intraphagosomal environment has been considered nutrient deficient. This has been deduced indirectly from various auxotrophic mutants attenuated for growth due to the limited availability of nutrients in the phagosomal compartment. Most amino acid auxotrophs of MTB are attenuated (Gordhan et al., 2002; Parish, 2003; Sambandamurthy et al., 2005; Sampson et al., 2004) suggesting a restriction either in terms of abundance of, or accessibility to amino acids (Seth and Connell, 2000). The exception seems to be the methionine auxotroph of M. bovis BCG which is not compromised for survival in mice (Wooff et al., 2002). Methionine has also been suggested to be the main sulphur source for pathogenic mycobacteria in vivo (Wheeler et al., 2005). These finding are analogous to observations in Brucella spp., which suggested that although the synthesis of other amino acids is essential for intracellular multiplication, a methionine mutant was not found in a screen for growth inhibition in macrophages (Kohler et al., 2002). Conflicting studies from other intracellular pathogens such as Legionella pneumophila suggest that amino acids constitute the main source of carbon and energy (George et al., 1980). However, this may reflect vacuole differences which are subject to bacterial manipulation (Muñoz-Elias and McKinney, 2006), or, as in the case of L. pneumophila, amino acid auxotrophy may be an in vitro phenomenon since it possesses the synthetic gene complement for several amino acids which may account for its ability to persist in diverse environments (Chien et al., 2004).

The broad metabolic adjustments mediated by RelMtb-dependent accumulation of ppGpp in vitro in response to starvation (Primm et al., 2000) have been likened to long term persistence in vivo (Dahl et al., 2003). A mutant of MTB defective for the RelMtb-mediated stringent response was not attenuated in macrophages (Primm et al., 2000), but was severely impaired in sustaining chronic infection, suggesting that nutrient deprivation is reflective of host containment within noncavitating granulomas rather than within the macrophage. Concentrations of vacuolar iron are also thought to be higher than previously believed (Fontan et al., 2008; Wagner et al., 2005). In general though, the expression signature for intraphagosomally residing MTB finds many genes required for the synthesis of amino acids,
purines, and pyrimidines up regulated, indicating that bacteria are probably not able to obtain these components from the phagosome, where phosphate and sulphate are also considered to be limiting (Fontan et al., 2008; Rachman et al., 2006a).

Genes involved in β-oxidation, the glyoxylate cycle and gluconeogenesis which are reportedly upregulated (Fontan et al., 2008; Rachman et al., 2006a; Schnappinger et al., 2003; Timm et al., 2003) further underscore the lipid-rich nature of the replicative vacuole of MTB. The absence of the phosphotransferase system for carbohydrate uptake and the detection of only five sugar permeases further reinforces the notion that the phagosome does not provide an environment rich in diverse sugars (Titgemeyer et al., 2007).

Finally, the induction of a set of genes which are involved in cell wall and cell membrane components (Fontan et al., 2008; Rachman et al., 2006a) suggests that perturbation of the cell envelope is a major intracellular stress imposed by the macrophage, with lipid metabolism allowing for the remodeling and maintenance of this periphery which is actively engaged in modulating the immune system.

1.3.4 Cell envelope shedding

After infection of macrophages, MTB undergoes several changes in secretion and expression of surface components. In respect of energy consumption, the continual production and release of lipidated cell wall constituents is very expensive although justifiably beneficial to the bacilli as it manipulates the host response to influence disease progression. These biologically active lipid components may play a role in vacuole modulation (Fratti et al., 2003) or immune modulation (Brennan, 2003). Included in the latter is the class of methyl-branched fatty lipids (Kolattukudy et al., 1997; Minnikin et al., 2002) thought to be important for virulence (Cox et al., 1999; Sirakova et al., 2001). The biosynthesis of the surface-exposed lipids, phthiocerol dimycocerosates (PDIMs), and glycosylated phenolphthiocerol esters is unique to the virulent strains of mycobacteria (Azad et al., 1997; Reed et al., 2004). The complex synthesis and transport of this wax has been characterised in detail. Four of the six multifunctional proteins of the modular polyketide synthase (PKS) system show selectivity
for methylmalonyl-CoA, allowing the introduction of the characteristic methyl branches into
the PDIM molecule (Fernandes and Kolattukudy, 1997; Jain and Cox, 2005; Rainwater and
Kolattukudy, 1985; Trivedi et al., 2005). Several of these biosynthetic genes are significantly
upregulated in macrophages (Fontan et al., 2008). PDIM production has been shown to
modulate the early immune response to infection in a tissue-specific manner (Cox et al., 1999;
Rousseau et al., 2004). Hepta- and octamethyl phthioceranic acids and hydroxyphthioceranic
acids are the major acyl constituents of sulfolipids (SL) (Sirakova et al., 2001), where SL-N, a
precursor of the major sulfatide of MTB, SL-1, is highly immunogenic (Gilleron et al., 2004).

Of recent interest is the synthesis of the class of related PDIM lipids, phenolic glycolipids,
which confers on a subset of MTB isolates belonging to the W-Beijing family, the ability to
inhibit the release of key inflammatory effector molecules by cells of the host’s innate
immune response (Reed et al., 2004; Tsenova et al., 2005). However, it has recently been
shown that although PGL production contributes to the regulation of the host cytokine
response, a global suppressive effect amongst all PGL-producing W-Beijing strains is not
exhibited, but instead is dependent on the background of the strain in which it is produced
(Sinsimer et al., 2008). This finding argues against earlier speculation that the associations
between bacterial lineage and disease phenotype could be explained by the presence or

Anti-inflammatory signals can also be potentiated by TLR-2 dependent signaling of the 19-
kDa lipoprotein (Noss et al., 2001) and binding of mannose-capped lipoarabinomannan to
DC-SIGN (Geijtenbeek et al., 2003). Trehalose mycolates, mycolic-acid containing
glycolipids, positively and negatively influence granulomatous inflammation (Geisel et al.,
2005) based on the stereochemistry of the cyclopropane modification (Rao et al., 2005; Rao et
al., 2006). These subtle differences in structure fine-tune the immune pathology to a level
believed to be appropriate to the survival and persistence strategies of the bacteria (Russell,
2007).

These reports are all consistent with notion that MTB plays an active part in disease
progression. Indeed, varying features of the dynamic cell envelope, whose composition is
controlled to a degree by the intramembrane protease \textit{Rv2869c} (Makinoshima and Glickman,
translates into the differential infection outcomes observed by different strains which elicit different immune responses (Manca et al., 2004).

1.4 Carbon metabolism in MTB

1.4.1 Interpreting carbon usage in vivo through metabolic pathway assessment

MTB is an intracellular pathogen, although this organism shows no obligate parasitism as it is a prototroph possessing a flexible metabolic arsenal. Niche-specific adaptation of mycobacteria has evolved to allow optimal usage of available nutrients from the surrounding environment. The substrates define the metabolic pathways harbored by the bacterium for acquisition and assimilation of the respective nutrients. To this end, MTB is capable of oxidizing a variety of carbon substrates including carbohydrates, hydrocarbons, alcohols, ketones and carboxylic acids (Wheeler, 2005). The diverse pathway infrastructure necessary for catabolism of these sugars and fatty acids is evidenced in the gene complement in MTB for β-oxidation, glycolytic and pentose phosphate pathways and the TCA and glyoxylate cycles (Cole et al., 1998). The genes encoding fatty acid β-oxidation enzymes show a remarkable degree of paralogous expansion: for example, there are 36 paralogues each of FadD and FadE, involved either in fatty acid degradation or in the biosynthesis of acyl-CoA substrates (Cole et al., 1998; Saxena et al., 2003). This functional diversification may be a mechanism by which MTB ensures its ability to utilise the variety and quantity of mammalian fatty acids available within the host (Hiltunen and Qin, 2000). The topological organization of the large MTB metabolic network is also a testimony to the remarkable diversification evident within the MTB metabolome (Verkhedkar et al., 2007). A functional consequence is a large number of alternate routes, augmenting redundancy and flux plasticity (Almaas et al., 2005). In effect, the more reactions a metabolic network possesses, the stronger the network-induced redundancy, and the smaller the core of essential reactions. In this way, MTB maintains the integrity and robustness of its network, allowing for increased adaptive versatility required for occupancy of the organism within the differing host environments.
The observed abundance of lipolytic genes, in conjunction with the earlier studies which found mycobacteria isolated from infected mouse lung can readily metabolise both odd- and even-chain fatty acids (Bloch and Segal, 1956; Wheeler and Ratledge, 1988), support the proposition that these may be the dominant substrates during MTB infection. However, this may be subject to tissue (niche)-specific variation (Neyrolles et al., 2006; van der Wel et al., 2007) defined by the stage of infection (Boshoff and Barry, 2005). Various studies have revealed unique transcriptome signatures of MTB in human granulomas depending on the site of sample isolation (Fenhalls et al., 2002; Rachman et al., 2006b; Timm et al., 2003). These findings caution against using information of this type to make broad generalizations regarding the in vivo physiology of MTB.

Consistent with a dependence of MTB on lipid metabolism, however, is the fact that ICL, which catalyzes the first step of glyoxylate cycle, is essential for growth and persistence of this organism in macrophages and mice (Muñoz-Elias and McKinney, 2005). However, the murine model does not mimic the environmental heterogeneity displayed in humans which is highlighted by relatively little overlap in gene expression profiles between the two (Boshoff and Barry, 2005; Rachman et al., 2006b). Specifically icl gene transcript detection is extremely variable, and furthermore, icl transcript appears to be entirely absent from the central necrotic area and transition zones of human necrotic granulomas (Fenhalls et al., 2002).

Although prevalent, lipids, in all likelihood, are not the exclusive carbon source for MTB in vivo. During the acute stage of infection, a putative high affinity disaccharide transporter (lpgY sugABC) is essential for virulence of MTB in mice (Sassetti and Rubin, 2003) and in chronic infection, metabolically active bacilli transcribe genes for carbohydrate and lipid utilization (Talaat et al., 2007), suggesting that MTB may utilise both carbon sources. In other organisms, species-specific differences in this regard have been observed. For example, in Azotobacter vinelandii, acetate induces fatty acid dependent inhibition of glucose uptake and glycolysis (Tauchert et al., 1990), E. coli preferentially uses glucose which represses the glyoxylate shunt (Walsh and Koshland, 1985), whereas Corynebacterium glutamicum can grow on a mixture of glucose and fatty acids with the glyoxylate shunt predominantly fulfilling the anaplerotic functions (Wendisch et al., 2000). The impact of substrate affinity
and regulatory control would need to be assessed for MTB grown on a mixture of lipids and carbohydrates. It has been suggested that the dispensability of key glycolytic enzymes for *in vivo* growth (Sassetti and Rubin, 2003) is due to carbohydrate intermediates, such as glucose-6-phosphate, serving as precursors for biosyntheses (Muñoz-Elias and McKinney, 2006). This view is supported by the essentiality of pyruvate kinase (*pykA*), which catalyzes the last step in glycolysis, *in vitro* (glucose carbon source) but not *in vivo* (Sassetti *et al.*, 2003; Sassetti and Rubin, 2003). A single nucleotide polymorphism (SNP) in this gene that renders pyruvate kinase inactive is present in some members of the MTB complex, further substantiating the importance of lipids for growth *in vivo* (Keating *et al.*, 2005). Consistent with this notion is the induction of *pckA*, which encodes the gluconeogenic rate-limiting enzyme, phosphoenolpyruvate carboxykinase, which converts fatty acids into sugars (Schnappinger *et al.*, 2003). The avirulent nature of a mycobacterial mutant defective for this gene (Collins *et al.*, 2002a; Liu *et al.*, 2003) reinforces the concept that carbohydrates are used for biosynthetic rather than catabolic purposes, since glucose-induced *pckA* downregulation (Liu *et al.*, 2003) would afford a pyruvate carboxylase (PCA) bypass in the presence of carbohydrates (Fig. 1).

Upregulation of *icl* and *pckA* (Schnappinger *et al.*, 2003; Timm *et al.*, 2003) might not only signal fatty acid metabolism but also the possible oxidation of acetyl-CoA to CO$_2$ by the PEP-glyoxylate cycle, active under glucose limitation, possibly compensating for higher NADPH formation on alternative substrates (Fischer and Sauer, 2003; Muñoz-Elias and McKinney, 2006). The detection of the competing enzyme, isocitrate dehydrogenase (Fischer and Sauer, 2003), indicates that the TCA’s biosynthetic role would be provisionally functional. Indeed, it has been proposed that MTB operates in the half cyclic mode leading to α-ketoglutarate (2KG) via the oxidative branch and succinate via the reductive branch (Muñoz-Elias and McKinney, 2006; Tian *et al.*, 2005a) quenching the bacterium’s biosynthetic demands rather than providing metabolic energy. This may be a consequence of the disjunction at the level of conversion of 2KG to succinyl-CoA (Fig. 1)(Tian *et al.*, 2005a; Tian *et al.*, 2005b) – a characteristic feature of some microbes that are adapted to low oxygen conditions (Pitson *et al.*, 1999). These two branches in MTB are metabolically linked by either the glyoxylate shunt or a novel, variant bypass pathway via α-ketoglutarate decarboxylase and succinic semialdehyde dehydrogenase (Tian *et al.*, 2005a). However, the existence of other bypass
pathways acting under niche-specific conditions such as the γ-aminobutyric acid (GABA) shunt or a α-ketoglutarate oxidoreductase cannot be excluded and needs investigation. Importantly, only this latter option would generate succinyl-CoA as an intermediate in the canonical TCA cycle, in an energy efficient manner. Alternatively, pools of succinyl-CoA for methionine, diaminopimelate and heme biosynthesis would need to be derived from succinyl-CoA synthetase in an energy-dependent manner (Carrillo-Castaneda and Ortega, 1970) or from propionyl-CoA via the methylmalonyl-CoA pathway (described in section 1.5.1). The described bypass pathway in MTB (Tian et al., 2005a) is energetically unfavorable with respect to succinyl-CoA generation, with the favored selection for the alternate routes to succinyl-CoA and their contribution to in vivo metabolism largely unknown.
MDH/ MQO
FUM
KGD
ACN
CIT
ICD
SDH
SCS
ICL
pyruvate
PEP
PPS
PYK
PDHC
acetyl-CoA
oxaloacetate
malate
FUM
fumurate
SDH
succinate
α-ketoglutarate
succinyl-CoA
MDH/ MQO
Glyoxylate cycle
acetyl-CoA
PEP
PCA
MEZ
PPC
PCK
FA β-oxidation
propionyl-CoA
Methylcitrate cycle
TCA cycle
Methylmalonyl pathway
citrate
isocitrate
acetyl-CoA
KGD
ICD
ACN
CIT
PCA
MLS
glyoxylate
ICL
glyoxylate
Fig. 1 Anaplerotic pathways in MTB.
Pathways feeding into the TCA cycle which are present in MTB are denoted by solid arrows and pathways not present in MTB are denoted by dashed arrows, namely phosphoenolpyruvate (PEP) synthase (PPS) and PEP carboxylase (PPC). Pyruvate is produced from malate by malic enzyme (MEZ) or from oxaloacetate by the sequential action of PEP carboxykinase (PCK) and pyruvate kinase (PYK); coupled decarboxylation of pyruvate by the pyruvate dehydrogenase complex (PDHC) yields acetyl-CoA. Anaplerosis during carbohydrate catabolism is by carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase (PCA). Previous studies have established the importance of the glyoxylate cycle for anaplerosis during fatty acid (FA) catabolism by MTB (Muñoz-Elias and McKinney, 2005). Glyoxylate cycle enzymes are the isocitrate lyases (ICL1/ICL2) and malate synthase (MLS). Anaplerosis during odd-chain FA β-oxidation by the methylmalonyl pathway occurs at the succinate dehydrogenase (SDH) branch point of the tricarboxylic acid (TCA) cycle adapted from Tian et al (2005). CIT, citrate synthase; ACN, aconitase; ICD, isocitrate dehydrogenase; KGD, α-ketoglutarate decarboxylase; SSADH, succinic semialdehyde dehydrogenase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; MQO, malate:quinine oxidoreductase; SCS, succinate synthase.

1.4.2 Regulation of carbon metabolism in MTB

MTB’s regulatory repertoire includes 190 transcriptional regulators (Cole et al., 1998), which collectively coordinate gene expression necessary for pathogenesis. Partial characterization of a few two-component regulatory systems such as DosR/S/T (Malhotra et al., 2004), RegX3-SenX3 (Parish et al., 2003), MprA-MrpB (Zahrt and Deretic, 2001) and PhoP-PhoQ (Walters et al., 2006) and a few selective sigma factors (Ando et al., 2003; Sun et al., 2004) have implicated these systems in virulence. However, the molecular mechanisms of how MTB senses environmental stresses and induces metabolic adaptation remain largely unknown. Recent studies indicate that in the absence of SoxR regulatory protein and FNR (fumarate nitrate regulator), MTB possesses an Fe-S cluster transcription factor, WhiB3, and heme proteins DosS and DosT, sensors which detect environmental redox signals such as O₂, NO and fluctuations in the intracellular redox state to promote bacterial persistence (Kumar et al., 2007; Singh et al., 2007a). The induction of the DosR regulon in palmitate (Boshoff et al., 2004) and the essentiality of WhiB3 for growth on various carbon sources, including TCA intermediates and acetate (Singh et al., 2007a), stresses the coupling of the intracellular redox
environment to central and fatty acid metabolism. For instance, in glucose limited cultures of *E. coli*, the regulation of key enzymes of the TCA cycle is mediated by FNR and ArcA, O$_2$-sensing regulators (Shalel-Levanon *et al.*, 2005).

Contribution to the global control of carbon metabolism in MTB in response to the many extracellular and intracellular signals through a second messenger system based on cyclic nucleotide monophosphates (cNMP) has been reported (McCue *et al.*, 2000). In *E. coli*, the role of cAMP signal transduction in mediating catabolite repression has been well characterised with the adenylate cyclase catalysing the synthesis of cAMP, which then transduces the signal by binding cAMP receptor protein (CRP) and activating it as a transcription factor (de Crombrugghe *et al.*, 1984; Kolb *et al.*, 1993). MTB has 15 putative nucleotide cyclases and eight orthologues of cAMP receptor protein (CRP)-like transcriptional regulators (Dass *et al.*, 2008; McCue *et al.*, 2000). Rv3676 is one such CRP-like transcriptional regulator described in MTB (Bai *et al.*, 2005). Deletion of this gene leads to attenuation in macrophage infections and in the mouse model of TB (Rickman *et al.*, 2005). The lack of Rv3676 function in *M. bovis* BCG has also been suggested to be one of the additional reasons for its attenuation (Brosch *et al.*, 2007; Spreadbury *et al.*, 2005). Together, these observations suggest that several important genes are under cAMP regulation.

In a recent study, the relative distribution of flux through central metabolism under glucose limitation was dominated by cAMP-dependent catabolite repression (Nanchen *et al.*, 2008). Importantly, flux through the PEP-glyoxylate cycle is controlled by intracellular cAMP levels, actively induced by the cAMP-CRP complex. In *C. glutamicum*, the ICL-encoding gene, *aceB*, is under the control of the CRP homologue, GlxR (glyoxylate bypass regulator) (Kim *et al.*, 2004), which is highly homologous to Rv3676 (75% amino acid identity). A potential CRP binding site was identified 279 bp upstream of the MTB glyoxylate cycle gene, *icl* (Spreadbury *et al.*, 2005), although the cognate regulatory factor remains undefined since computational methods for identifying potential regulon members of the Rv3676 did not include *icl* as a candidate (Bai *et al.*, 2005). An alternative theory stems from the observation that malate dehydrogenase (*mdh*) expression levels were increased by cAMP in shaking low oxygen cultures (Gazdik and McDonough, 2005). MDH converts malate to oxaloacetate and it
is speculated that increased levels are necessary to drive the forward reactions of the glyoxylate cycle and thereby, indirect regulation of glyoxylate cycle metabolism by cAMP is achieved. In *E. coli*, glyoxylate cycle gene expression is controlled by the IclR repressor which regulates the *aceBAK* operon (Sunnarborg *et al.*, 1990), encoding isocitrate dehydrogenase (ICD) kinase (*aceK*), and both glyoxylate cycle enzymes, ICL (*aceA*) and MLS (*aceB*). At the branch-point of the TCA cycle and the glyoxylate cycle, flux is predominantly controlled through the reversible inactivation of ICD by phosphorylation, catalysed by ICD kinase (LaPorte *et al.*, 1984; Walsh and Koshland, 1985). A bioinformatics search of the MTB proteome revealed that Rv1719 contains an N-terminal helix-turn-helix DNA binding motif followed by an IclR-type effector binding domain (Pfam PF01614), and along with reports that MTB possesses an ICD kinase (Singh and Ghosh, 2006) suggest that MTB may have a similar mechanism of control. Metabolic control analysis (MCA) predicted that reducing kinase activity by 30% would increase the amount of dephosphorylated (active) ICD sufficiently to diminish flux through the glyoxylate cycle (Singh and Ghosh, 2006), consistent with the low affinity of ICL for the competing isocitrate substrate (K_m=188 µM) (Gould *et al.*, 2006) relative to ICD-1 (K_m=10µM) and ICD-2 (K_m=20µM) (Banerjee *et al.*, 2005). The mechanisms employed to regulate gene expression are varied and complex and defining the precise mechanisms for directing carbon flux in MTB requires further investigation. Recent work identified a TetR-type regulator, KstR (ketosteroid regulator) which is induced in response to growth on palmitate, which was proposed to regulate the uptake and utilization of a variety of lipids, including cholesterol; however, it also influences genes of the pentose phosphate pathway and glycerol metabolism (Kendall *et al.*, 2007a; Van der Geize *et al.*, 2007).

### 1.4.3 The sources and types of nutrients *in vivo*

Discrete microanatomical niches exist within the human host and sources of nutrients are likely to be varied and vast. Proliferation of MTB either within the extracellular caseous matter of cavities (Grosset, 2003; Lenaerts *et al.*, 2007) or within permissive phagocytes such as that witnessed at the margins of liquefied lesions in lungs (Kaplan *et al.*, 2003) and in
follicular structures in the peripheral rim of closed necrotic noncavitating granulomas (Ulrichs et al., 2004), attests to the bacilli’s exquisite ability to actively harness intracellular carbon sources, so much so that they have been described as ‘fat’ (Garton et al., 2008) and to mobilise these extracellular sources into internal reserves of storage lipids, known as triacylglycerols (TAG). These lipids are thought to provide a carbon and energy source during periods of latency and transmission when stored TAG is hydrolysed following, for instance, PE30 lipase induction (Schnappinger et al., 2003). The high global prevalence of strains of MTB of the W-Beijing lineage and their apparent advantage over other lineages (Barczak et al., 2005; Reed et al., 2007) has been attributed in part to the striking accumulation of TAG in these strains as a result of a diacylglycerol acyltransferase (TAG synthase; Rv3130c), a member of the DosR regulon which is constitutively expressed in these strains (Reed et al., 2007). TAG accumulation has been found in bacteria obtained from organ lesions (Garton et al., 2002; Garton et al., 2008) which might represent TAG stored during dormancy or produced in the expanding granuloma from the fatty acids released from the degrading host tissue (Daniel et al., 2004). The exogenous fatty acids may form TAG by direct incorporation or elongation using de novo synthesised fatty acids from the C2 units generated by β-oxidation of host fatty acids (Garton et al., 2002; Sirakova et al., 2006). Fifteen TAG synthases have been identified in the MTB genome. Many are co-transcribed with lipases that are actively involved in making fatty acids available from several sources, including: (i) host cellular or membrane lipids (Jackson et al., 1989; Mason et al., 1972); (ii) bacterial phospholipid turnover (Garton et al., 2002); (iii) release of long fatty acid chains from polyketide synthases (Asselineau et al., 2002); (iv) accumulated C24 and C26 fatty acids due to possible FASII down-regulation in macrophages in response to acidic pH (Daniel et al., 2004; Fisher et al., 2002). Therefore, most TAG synthases are likely to display broad substrate specificity induced under the influence of different host factors (Alvarez and Steinbuchel, 2002). The rich source of energy that TAG, sourced either from the host or synthesized by the bacilli, may provide to bacteria in a predominantly low metabolic state, in combination with the essential ICL requirement for persistence, strongly implicates TAGs as a necessary component of the physiology of MTB for long-term survival. This is marvelously exemplified by the finding that MTB gravitates to extra-pulmonary fatty deposits, where it sequesters host fatty acids into
dense TAG droplets and persists for years undetected by immune surveillance (Neyrolles et al., 2006). Similarly lipid-rich foamy macrophages may constitute a reservoir for persisting mycobacteria which display intracytoplasmic lipid inclusions when freed from the phagosome into lipid droplets, favouring internalization of cellular host lipids (Peyron et al., 2008).

Lung surfactant ingested by alveolar macrophages (Grabner and Meerbach, 1991) also contains other complex lipids such as dipalmitoyl phosphatidylcholine, a membrane phospholipid, which MTB is capable of metabolizing (Muñoz-Elias and McKinney, 2005) using phospholipase C (plc) to produce phosphorylcholine and diacylglycerol (DAG) (Johansen et al., 1996), with the latter potentially undergoing further acylation to TAG. In addition, phospholipases C are required for releasing fatty acids from the host phospholipids, which may then be used as a carbon source through β-oxidation and the glyoxylate cycle, with the joint cooperation between plc and icl evident in their combined requirement for virulence (Muñoz-Elias and McKinney, 2005; Raynaud et al., 2002).

Other intraphagosomal lipid metabolizing pathogens include Rhodococcus equi (Wall et al., 2005), Candida albicans (Lorenz and Fink, 2002) and Salmonella enterica serovar typhimurium (Fang et al., 2005), which rely on a functional glyoxylate cycle like MTB for catabolising the concentrated lipid source in this compartment. The induction of fatty acid β-oxidation in E. coli has recently been shown to be dependent on the RelA-mediated stringent response as part of the expanded repertoire of metabolic genes induced by ppGpp to remediate nutrient stress (Traxler et al., 2008). This finding further informs the complex composition of the phagosome environment which is in stark contrast to the improved nutritional status of the cytosol, reportedly abundant in glycogen (Ripio et al., 1997), oligopeptides, aminosugars and amino acids (Chatterjee et al., 2006). Consistent with this, cytosolic pathogens such as Shigella flexneri (Lucchini et al., 2005) and Listeria monocytogenes (Chatterjee et al., 2006; Chico-Calero et al., 2002) are icl-deficient. Intracellular Legionella pneumophilia scavanges host amino acids and potentially carbohydrates from the host (Bruggemann et al., 2006). Escape into the energy-rich cytosolic environment is documented for MTB where the bacilli multiply more rapidly than their vacuolar counterparts (van der Wel et al., 2007). As observed in Candida albicans, progression to systemic disease may be dependent on glycolysis (Barelle et al., 2006).
The usage of available nutrient sources is clearly dependent upon the location of MTB. It is conceivable, for instance, that bacteria escaping the granuloma and persisting in healthy lung tissue (Hernandez-Pando et al., 2000; Opie, 1927) may use the rich source of cholesterol in pulmonary exudates, a conclusion consistent with the metabolic pattern that emerged from \textit{in vivo} non-replicating persistent type bacilli in sputum (Garton et al., 2008). Mounting evidence that cholesterol is an important carbon source for MTB has emerged in recent years. Uptake and catabolism of this complex sterol has been demonstrated and accessing this unique carbon source has been shown to be essential to the survival success of MTB (Joshi et al., 2006; Pandey and Sassetti, 2008; Van der Geize et al., 2007). Gaining entry into host cells by promiscuous binding to multiple surface receptors laden in cholesterol-rich domains of the plasma membrane (Gatfield and Pieters, 2000) ensures a higher cholesterol fraction within vesicles (Mason et al., 1972). Together with cholesterol-rich glycosphingolipid fusion to mycobacterial vacuoles (Russell et al., 1996), this `carbon collateral’ may provide a distinct advantage to MTB.

An interesting alternative is that mycobacteria may have evolved to utilise lipids in order to neutralise the free fatty acids produced by macrophages in antimicrobial defense (Akaki et al., 2000; Coonrod et al., 1989), in addition to their use as an energy source. In support of this hypothesis, free fatty acids are reportedly inhibitory to lipogenesis (Ferdinandus and Clark, 1969), and toxic free fatty acids can be incorporated into non-toxic triglyceride (McCarthy, 1971). This raises fundamental questions as to the established reasons for essentiality of certain genes involved in this process.

### 1.5 Propionate metabolism

The broad selection of substrates available to MTB and the respective complementing biochemical pathways for breakdown thereof have not been fully elucidated and are the focus of much ongoing research. For example, the established importance of \(\beta\)-oxidation and the glyoxylate cycle in virulence of several pathogenic microorganisms, including MTB, is a prerequisite for appreciating the degree of reliance on lipids as a carbon source \textit{in vivo}. 
Similarly, the attenuation observed for MTB defective in cholesterol uptake places into context the importance of this sterol as a carbon source. This leads to the actualization of a common thread between these essential converging pathways, namely, the potential precursors of propionyl-CoA. Metabolism of this intermediate is an aspect of MTB physiology which remains to be fully characterised.

Cellular generation of propionyl-CoA can stem from several different processes including thioesterification of propionate, catabolism of the branched-chain amino acids isoleucine, valine and methionine, decarboxylation of succinate and catabolism of odd- and branched-chain fatty acids. The cytotoxic effects of propionyl-CoA accumulation is associated largely with interference of pyruvate oxidation, competitive with coenzyme A (CoASH) (Brock et al., 2000; Brock and Buckel, 2004; Maerker et al., 2005; Maruyama and Kitamura, 1985). These effects encourage efficient clearance of this metabolite by channeling it as a carbon and energy source.

In mammalian tissue, propionate is physiologically derived from intestinal fermentation (Bergman, 1990) or from methyl-branched fatty acids, phytanic acid and the pristanic acid derivative, both present in the human diet (Verhoeven and Jakobs, 2001). Corynebacterium species (McKenna and Kallio, 1971) and mycobacterial species (Cox et al., 1972; Sakai et al., 2004) are capable of oxidizing such branched isoprenoid alkanes, where pristane is oxidised to pristanic acid which is then esterified with CoA, and after several successive cycles of β-oxidation, releasing three molecules of propionyl-CoA. The presence of pristane in human tissue (Avigan et al., 1967) might make this a relevant source of propionyl-CoA in MTB. Cholesterol is another potentially important source of propionyl-CoA in vivo yielding two molecules of propionyl-CoA per molecule of cholesterol catabolised by β-oxidation of the branched side chain at C17, and the catabolism of rings A and B (Van der Geize et al., 2007). Potentially, breakdown of these host derived lipids, and utilization of free fatty acids, C5 ketone bodies and host protein (Ibrahim-Granet et al., 2008), along with intracellular recycling of select MTB cellular components, may yield propionyl-CoA levels which demand its metabolism to be a pivotal facet of MTB physiology.
1.5.1 Characterised pathways for propionyl-CoA catabolism

There are several pathways characterised for the oxidation of propionate. All of the described pathways start with propionyl-CoA, which is synthesised by a tightly regulated ATP-dependent acyl-CoA synthase, in keeping with CoA homeostasis (Garrity et al., 2007; Palacios et al., 2003). Propionyl-CoA may undergo α-oxidation in the acryloyl-CoA pathway, β-oxidation by the 3-hydroxypropionate pathway, α-carboxylation via the methylmalonyl-CoA pathway, reductive carboxylation by the crotonyl-CoA pathway or Claisen condensation by the 2-methylcitrate, 2-hydroxyglutarate and citramalate pathways (Textor et al., 1997; Wegener et al., 1968b). The ultimate fate of activated propionyl-CoA is the production of either acetyl-CoA (non-randomising) or succinyl-CoA (randomising) as end-products of these catabolic pathways, entering the TCA cycle at the citrate synthase or succinate dehydrogenase steps, respectively (Fig. 1).

The best studied pathway is the route for propionate metabolism in higher eukaryotes via methylmalonyl in which propionyl-CoA is carboxylated to (S)-methylmalonyl-CoA, which is racemised to the (R)-enantiomer. The carboxylation of propionyl-CoA can be catalysed by distinct biotin-dependent enzymes, the propionyl-CoA:oxaloacetate transcarboxylase which functions in the reversible decarboxylation of propionyl-CoA in Propionibacterium shermanii (Hall et al., 2004), and propionyl-CoA carboxylases (PCC) which catalyse only the irreversible carboxylation between propionyl-CoA and (S)-methylmalonyl-CoA (Buckel et al., 2005). A biotin-independent methylmalonyl-CoA decarboxylase has also been identified (Haller et al., 2000). Isomerisation is mediated by the coenzyme B₁₂-dependent MCM to succinyl-CoA.

In animals and humans, propionate is oxidised by the methylmalonyl pathway which provides energy and removes toxic propionyl-CoA (Brock and Buckel, 2004). This pathway has been described in a number of bacteria, including Pseudomonas butanovora (Doughty et al., 2006), Sulfolobus solfataricus (Chong et al., 2007), Pelotomaculum thermopropionicum (Kosaka et
al., 2006), *Streptomyces cinnamonensis* (Vrijbloed et al., 1999), MSM (Stjernholm et al., 1962) and *M. vaccae* (Vestal and Perry, 1969).

The mutase enzyme also catalyses the reverse metabolic direction in a number of organisms (Haller et al., 2000), which may link succinate to polyketide synthesis (Hunaiti and Kolattukudy, 1984; Valentin and Dennis, 1996; Vrijbloed et al., 1999). *P. shermanii* employs the pathway for propionate formation in order to dispose of reducing equivalents in glucose or glycerol fermentations (Allen et al., 1964). In *Saccharopolyspora erythraea* the MCM provides methylmalonyl-CoA subunits for erythromycin production in oil-based fermentations, although it acts in the reverse direction in carbohydrate-based fermentations (Reeves et al., 2006, 2007).

### 1.5.2 The adenosylcobalamin-dependent methylmalonyl-CoA mutase reaction

The MCM enzyme catalyses a 1,2-intramolecular rearrangement of the thioester moiety (X= COSCoA) using adenosylcobalamin (AdoCbl; coenzyme B$_{12}$) specifically as a cofactor.

![Diagram of the adenosylcobalamin-dependent methylmalonyl-CoA mutase reaction](attachment:reaction_diagram.png)

A highly reactive free deoxyadenosyl radical species and cob(II)-alamin are generated by hemolytic cleavage of a C-Co bond of the coenzyme that is weakened by steric repulsion due to a protein conformation change induced by substrate binding (Garcia-Viloca et al., 2004; Mancia et al., 1999). The crystal structure of MCM in complex with its substrate shows the active site is deeply buried inside a TIM barrel structure, which shields the biradical intermediates from solvent and oxidative interception (Mancia and Evans, 1998; Thoma et al., 2000). The rate of Co-C bond homeolysis is accelerated by a factor of $10^{11}$ in the ternary complex of enzyme, AdoCbl and substrate relative to the uncatalysed rate in solution, effectively lowering the activation free energy (transition state barrier) by 71.1 kJ/mol.
(Chowdhury and Banerjee, 2000). The deoxyadenosyl carbon radical abstracts a hydrogen atom from the methyl group of the substrate to initiate a free radical mechanism for a carbon skeletal isomerisation, followed by re-abstraction of the hydrogen atom from deoxyadenosine to form the product and regenerate the cofactor (Banerjee and Vlasie, 2002; Vlasie and Banerjee, 2004). The hydrogen transfer proceeds by a highly quantal tunneling mechanism (Dybala-Defrattyka et al., 2007; Garcia-Viloca et al., 2004), and together with the lowering of the activation barrier, contributes to the dramatic rate acceleration of the MCM reaction (Chowdhury and Banerjee, 2000; Garcia-Viloca et al., 2004). In the case of the MCM from \textit{P. shermanii}, the overall equilibrium for the rearrangement reaction favors succinyl-CoA formation by a factor of 3 (Meier et al., 1996; Thoma and Leadlay, 1998) in agreement with the methylene radical (methylmalonyl-CoA) having a slightly higher energy state than the more stable secondary methine radical (succinyl-CoA) (Chowdhury and Banerjee, 2000). Stabilization of the methylene radical by cob(II)alamin (Buckel et al., 2005) and partial proton transfer to activate the migrating thioester group (Smith et al., 1999) are proposed mechanisms thought to facilitate the MCM-catalyzed reaction by lowering the transition states that interconvert these radicals.

The central catalytic role of coenzyme B\textsubscript{12} is therefore essential for the mutase reaction. Availability of the adenosylated form of cobalamin is ensured by the protein adenosyltransferase (ATR), which participates in the \textit{de novo} synthesis of AdoCbl or on precursor cobinamide (incomplete corrinoid) salvaged from the environment (Escalante-Semerena, 2007). For an obligate pathogen such as MTB which possesses three vitamin B\textsubscript{12}-dependent enzymes (Cole et al., 1998; Dawes et al., 2003; Warner et al., 2007) accessing the ‘escorted’ cobalamin (Yamanishi et al., 2005) from mammalian tissues where the concentration is quite low (30-700nM with >95\% of B\textsubscript{12} bound ; (Hsu et al., 1966; Shevell and Rosenblatt, 1992) is probably limited. However, it is important to note that the obligate pathogen, \textit{M. leprae}, has retained the genes encoding two vitamin B\textsubscript{12}–dependent enzymes (MetH, MCM) despite having lost most of the cobalamin synthesizing genes through reductive evolution (Cole et al., 2001). Alternately \textit{in silico} analysis by Rodniov et al (2003) classified MTB as having a complete adenosylcobalamin biosynthetic pathway although no evidence of \textit{de novo} synthesis exists. Therfore, an energy saving alternative to \textit{de novo}
synthesis is that exogenous corrinoids (molecules containing the cobalt-containing cyclic tetrapyrrole known as the corrin ring) may be salvaged and transported into the bacterial cell where AdoCbl, or potentially adenosylcobinamide (AdoCba; the coenzymatic form whose lower axial ligand is not dimethylbenzimidazole (DMB)) is formed (Anderson et al., 2008).

ATR catalyses the reductive adenosylation of cobalamin and transfers adenosylcobalamin to MCM (Padovani et al., 2008). Binding of the cofactor is accompanied by a significant conformational change in which the lower axial ligand, DMB, is coordinated to the cobalt atom (a mode of attachment that is called ‘base-on’), is displaced from the cobalt, and then replaced by the histidine residue in the conserved DXHXXG motif of the mutase family (Marsh and Holloway, 1992), with this base exchange being named ‘base-off, His-on’ (Chowdhury et al., 2001). Impairments in the ATR-dependent conversion of apo- to holo-MCM result in no mutase activity, which, in humans, leads to methylmalonic aciduria (Leal et al., 2003), and in Methylobacterium extorquens lacking the glyoxylate cycle, inhibits growth on C\textsubscript{2} compounds since the alternate anaplerotic pathway is dependent on MCM (Korotkova and Lidstrom, 2004; Korotkova et al., 2005). Significantly, MCM is a crucial component of alternate pathways to the glyoxylate cycle for acetate assimilation in various organisms, such as the glyoxylate regeneration cycle of M. extorquens (Korotkova et al., 2002), the ethymalonyl-CoA pathway described for Streptomyces collinus (Han and Reynolds, 1997), Rhodobacter sphaeroides (Alber et al., 2006; Erb et al., 2007) and Rhodobacter capsulatus (Meister et al., 2005), and the citramalate cycle of Rhodospirillum rubrum (Berg et al., 2002), highlighting the importance of vitamin B\textsubscript{12} for growth of these organisms on acetate.

1.5.3 Anaplerosis by the methylmalonyl pathway

Anaplerosis is of Greek origin meaning ‘to fill up’, and refers to pathways that form intermediates that replenish the TCA cycle. Several organisms lacking the vitamin B\textsubscript{12}-dependent MCM (Ledley et al., 1991) or only the coenzyme B\textsubscript{12} (Huser et al., 2003; London et al., 1999), may use the methylcitrate cycle for the assimilation of propionate (Brock et al., 2001; Claes et al., 2002). This cycle was first discovered in alkane- and lipid metabolizing
yeasts (Tabuchi and Serisawa, 1975). The simple α-oxidation of propionate to pyruvate is achieved by the condensation of propionyl-CoA with oxaloacetate to yield (2S,3S)-methylcitrate, followed by isomerisation of this tricarboxylic acid to form (2R,3S)-2-methylisocitrate, which is cleaved to succinate and pyruvate. Studies with 13C-labeled propionate indicate that in E. coli, the pyruvate is further oxidised to acetyl-CoA and channeled into the anaplerotic glyoxylate cycle (Textor et al., 1997). In this system, the PEP synthase and the PEP carboxylase were unable to support growth in the absence of ICL (Textor et al., 1997; Wegener et al., 1969). A distinguishing feature between propionate oxidizing pathways has emerged in that E. coli, which cannot synthesise vitamin B12 de novo but possesses a functional MCM (Roth et al., 1996; Roy and Leadlay, 1992), when supplemented with vitamin B12, appeared to have a near zero glyoxylate flux when this pathway was used for propionate oxidation (Evans et al., 1993). In addition, blocking the methylmalonyl pathway at the succinate dehydrogenase level resulted in an absence of labeled glutamate as observed in control wild type cultures, strongly suggesting that this may be the result of the glyoxylate cycle that is now induced as described by Textor et al., shifting the flux away from isocitrate dehydrogenase in the absence of succinyl-CoA anaplerosis and minimizing the incorporation of label into α-oxoglutarate (Fig. 1). This raises the intriguing question as to whether the anaplerotic flux from succinyl-CoA from the methylmalonyl pathway is sufficient to support growth of E. coli in the absence of ICL if provided with exogenous vitamin B12. Significantly, similar findings have been reported in Pseudomonas and mycobacterial strains (Blevins and Perry, 1972). In this study, no ICL activity was detected for growth on propylamine (a propionyl-CoA precursor) or propionate. Furthermore, incorporation of 14CO2 into pyruvate confirmed the presence of the anaplerotic propionyl-CoA carboxylase–methylmalonyl-CoA pathway of propionate utilization. Interestingly, inhibition of the pyruvate dehydrogenase enzyme complex altered the amount of labeled pyruvate as compared to the uninhibited control, suggesting that flux through the methylcitrate cycle is disrupted by this manipulation, increasing flux through the methylmalonyl pathway. Indeed, much speculation may be made regarding pathway preference, given evidence that they may operate simultaneously (Evans et al., 1993). The greater incorporation of 14CO2 into pyruvate during propylamine oxidation relative to growth on propionate (Blevins and Perry, 1972)
suggests that metabolism by one pathway is preferred in respect to carbon source, or more specifically, ambient propionate concentration. This is illustrated in *Burkholderia sacchari* where disruption of the methylcitrate cycle is only relevant in terms of increasing poly-3-hydroxybutyrate-3-hydroxyvalerate (3HV) production (*i.e.* a mutation in the methylcitrate cycle results in more propionate availability for 3HV) at lower propionate concentrations (0.02%), whereas at increased propionate concentrations, oxidation by an as yet unidentified contributor pathway becomes significant (Bramer *et al.*, 2002). MSM does not require the methylcitrate cycle for growth on propionate media (Upton and McKinney, 2007) and although MTB relies on the methylcitrate cycle for growth *in vitro* (outside the living host), it is dispensable for survival *in vivo* (Muñoz-Elias *et al.*, 2006). These findings indicate the existence of alternate pathways for propionate metabolism in these mycobacterial species, consistent with the presence of orthologues specific to the methylmalonyl pathway in both MSM (Muñoz-Elias *et al.*, 2006) and MTB (Cole *et al.*, 1998; Fleischmann *et al.*, 2002).

Clearly, one notable difference between the methylmalonyl pathway and the alternate 2-methylcitrate cycle is the ability of the former to substitute for the glyoxylate cycle of the TCA cycle through the net generation of succinate rather than pyruvate (Fig. 1). This anaplerotic role of the MCM pathway seems to be exploited in several instances by organisms deficient for ICL, supporting growth on propionyl-CoA precursors such as C$_3$-C$_9$ monocarboxylic acids (Nieder and Shapiro, 1975). A mycobacterial sp. defective for the glyoxylate cycle due to a mutated MLS gene could grow on pristanic acid (Sakai *et al.*, 2004). Similarly deletion of the *glcB* gene encoding MLS in MSM did not affect growth on propionate as the sole carbon source (Upton and McKinney, 2007). However, in these cases little is known about the identity of all the propionate metabolizing pathways. It is possible that growth on propionate or propionyl-CoA derivative carbon sources in the absence of the glyoxylate cycle may be dependent on MCM although has not been established empirically. In humans, stimulating anaplerosis through the methylmalonyl pathway as a remedy for fatty acid oxidation disorders is widely acknowledged (Reszko *et al.*, 2003; Roe *et al.*, 2002b). Alternately, other propionate oxidation pathways which instead yield acetyl-CoA may be operating. In the absence of the glyoxylate cycle, the acetyl-CoA is channeled by pyruvate
carboxylase or PEP synthase / PEP carboxylase into the TCA cycle (Fig. 1) to support growth (Horswill and Escalante-Semerena, 1999). Further studies need to be done in order to elucidate the degree of importance that anaplerosis from these multiple donor pathways has in supporting growth on propionate-generating carbon sources in mycobacteria and other microorganisms.

The methylcitrate cycle is analogous to the first few steps of the TCA cycle utilizing the common enzymes, SDH, FUM, MQO and/or MDH to regenerate oxaloacetate from succinate in the methycitrate cycle. Aconitase, also common to the methylcitrate and TCA cycles, mediates only the rehydration of 2-methyl-cis-aconitate to (2R,3S)-2-methylisocitrate following dehydration of (2S,3S)-2-methylcitrate by MCD in the methylcitrate cycle. Furthermore, intermediates of the methylcitrate cycle inhibit isocitrate dehydrogenase of the TCA cycle (Brock, 2005; Cheema-Dhadli et al., 1975; Horswill et al., 2001; Plaut et al., 1975). Such interferences with the TCA cycle are proposed to diminish the rate of ATP synthesis providing a strong selective advantage for the autonomous methylmalonyl pathway in any organism intent on preserving energy (Buckel et al., 2005). This notion is strongly reinforced by the preservation of the component genes for the methylmalonyl pathway in M. leprae, an organism which has undergone large-scale reductive evolution (Cole et al., 2001).

1.6 The anabolic role of MCM in polyketide synthesis

In MTB, the lipid profile is influenced by the available carbon source (Brown et al., 2008; Jain et al., 2007; Pandey and Sassetti, 2008). Similarly the mycolic acid pattern of the actinomycete Rhodococcus erythropolis is affected by growth on various carbon sources. Specifically, no odd-numbered carbon chains were found in the cell unless grown on odd- or branched-chain carbon sources such as propionate, valerate, nonane, undecane or pristane suggesting that the availability of propionyl-CoA precursor derived from these carbon sources is paramount in determining the mycolic acid pattern (Sokolovska et al., 2003). Conceivably, a similar reliance on available propionyl-CoA generating carbon sources for conversion to
methylmalonyl-CoA for use in methyl-branched chain fatty acid synthesis may be displayed in MTB (Fernandes and Kolattukudy, 1997; Jain and Cox, 2005; Rainwater and Kolattukudy, 1985; Trivedi et al., 2005). However in other organisms, using the citric acid cycle intermediate succinyl-CoA, MCM is a major source of methylmalonyl-CoA precursor for polyketide products (Dayem et al., 2002; Hunaiti and Kolattukudy, 1984; Reeves et al., 2007; Valentin and Dennis, 1996; Zhang et al., 1999). Significantly, disruption of this pathway has been shown to have an influence on secondary polyketide biosynthesis in other actinomycetes (Reeves et al., 2004; Valentin and Dennis, 1996; Vrijbloed et al., 1999).

In *S. cinnamonensis*, four routes to methylmalonyl-CoA have been described (Li et al., 2004; Vrijbloed et al., 1999; Zhang and Reynolds, 2001), two of which are proposed to function in MTB, namely isomerisation of succinyl-CoA catalysed by MCM and carboxylation of propionyl-CoA catalysed by PCC. Notably endogenous biotinylated proteins such as PCC are sensitive to reactive nitrogen intermediates released by activated macrophages which induce intermolecular disulphide formation and interfere with the proper assembly and function of the PCC oligomeric protein (Rhee et al., 2005).

Therefore, it is reasonable to speculate that in MTB, should methylmalonyl-CoA extender subunits necessary for methyl-branched fatty acid synthesis become limiting as a result of PCC inhibition or failure to derive sufficient precursor propionyl-CoA from niche-specific carbon sources in vivo, compensation by the anabolic succinyl-CoA→methylmalonyl-CoA reaction of MCM would maintain the methylmalonyl-CoA metabolite pool. This may be relevant to the survival of MTB as the methyl-branched lipid fraction of its cell wall may influence MTB physiology with respect to permeability, antigenic properties and susceptibility to bactericidal compounds (Camacho et al., 2001; Rousseau et al., 2004; Zuber et al., 2008). An increase in the contribution of MCM to methylmalonyl-CoA metabolite maintenance, concomitant with a decrease in flow of propionyl-CoA to methylmalonyl-CoA by PCC has been reported (Reszko et al., 2003). In effect, redistributing flux in response to such perturbations encountered in activated macrophages would achieve a higher order of cellular robustness, shown to be critical in maintaining steady-states of essential metabolites (Kim et al., 2007). In MTB, constant methylmalonyl-CoA levels may be maintained by regulatory modules associated with low degree metabolites resulting in functional clusters of
highly correlated reactions (Samal et al., 2006), in the absence of a MCM regulator, \textit{mutR} in MTB (Reeves et al., 2007). Notably, a strain overexpressing \textit{mutAB} was associated with a 90\% loss of the episomal construct carrying \textit{mutAB in vivo} which argues that perturbations of methylmalonyl-CoA metabolism are not well tolerated (Jain et al., 2007).

Potentially, PEP oxidation through the catabolic PEP-glyoxylate cycle (Fischer and Sauer, 2003) or oxidation of even-chain fatty acids to acetyl-CoA, which is assimilated through the glyoxylate cycle, may act to elevate endogenous succinate levels which may be used for anabolic polyketide synthesis through the MCM arm. Consistent with this, high pools of succinate are found in acetate grown cultures (Plasmeier et al., 2007; Wendisch et al., 2000). This would extend the importance of ICL beyond its classical role in the glyoxylate cycle to the provision of precursor succinate for methyl-branched fatty acid synthesis.

An inverse association between propionate catabolism through the methylmalonyl pathway and fumurate respiration has been proposed (Kosaka et al., 2006; Thompson and Zeikus, 1988). Fumurate respiration contributes to the generation of the PMF necessary for energy production in hypoxic nongrowing MTB (Rao et al., 2008). As a consequence, intracellular levels of succinate may be raised. It is therefore reasonable to postulate a co-regulation of fumurate respiration with methylmalonyl-CoA synthesis by MCM in MTB. Indeed, anaerobically induced fumurate reductase was found to produce succinyl-CoA required for methionine biosynthesis in the absence of \(\alpha\)-ketoglutarate dehydrogenase (Carrillo-Castaneda and Ortega, 1970). In MTB, the absence of \(\alpha\)-ketoglutarate dehydrogenase and the reductive TCA half-cycle (Tian et al., 2005a; Tian et al., 2005b) may similarly result in enhanced succinate and succinyl-CoA formation under conditions where fumurate reductase is derepressed, as in \textit{Helio bacter pylori} (Pitson et al., 1999).
2 AIMS and OBJECTIVES

Propionyl-CoA is an important intermediary high-energy metabolite, whose toxic accumulation may be defused through one of several routes. The main objectives of this study were to characterise the propionate oxidation pathways in MTB for growth on a variety of propionyl-CoA-generating carbon sources and to determine the functional relationship between these pathways \textit{in vitro}, in order to gain a greater understanding of the contributions of each pathway to MTB pathogenesis \textit{in vivo}. 
3 MATERIALS and METHODS

3.1 Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are detailed in Table S. 1. Wild type MTB (strain H37Rv, ATCC 25618) and derivatives were grown standing at 37°C in Middlebrook 7H9 media (Merck) supplemented with 0.2% glycerol, oleic acid-albumin-dextrose-catalase (OADC) enrichment (Merck) and 0.05% Tween 80. Sodium acetate, sodium propionate, valeric acid and heptadecanoic acid were purchased from Sigma. Bacteria grown on fatty acids were cultured in 7H9 media containing 0.5% albumin, 0.085% NaCl, 0.05% Tween-80 and sodium acetate, sodium propionate or valeric acid at a concentration of 0.1% (10 mM in all cases). Sodium propionate was also added to a final concentration of 0.2%. The pH of the valeric acid-containing medium was adjusted to 6.8 with 10M NaOH prior to use. In the case of heptadecanoic acid, a pre-warmed 0.2% stock solution of heptadecanoic acid was added to the media at a final concentration of 0.007% (0.25 mM). The poor solubility of heptadecanoic acid in water accounted for the lower final concentration of this carbon source. Unless otherwise indicated, vitamin B\textsubscript{12} supplement (cyanocobalamin (CNCbl)), Sigma) was included at a concentration of 10 \(\mu\text{g}/\text{ml}\). Hygromycin (Hyg) and kanamycin (Km) and streptomycin were used in MTB cultures at final concentrations of 50, 25 and 20\(\mu\text{g}/\text{ml}\) respectively. 3-Nitropropionate (3NP, Sigma) was used at a concentration of 0.1 mM (Muñoz-Elias and McKinney, 2005). Growth curves were monitored by measuring the optical density (OD\textsubscript{600}) of cultures using at least three independent biological replicates.

3.2 Construction and complementation of mutant strains of MTB

The DNA sequences of the genes under investigation and their flanking regions were obtained from http://genolist.pasteur.fr/TubercuList/. MTB genomic DNA containing the mutAB genes and flanking sequences was obtained from the BAC library (BAC Rv58 spanning region 1640681 to 172735) (Brosch et al., 1998) and digested with EcoRI to obtain a 7760bp
template which was cloned into p2NIL (Parish and Stoker, 2000) to form p2mutAB. An internal, 2342-bp region of mutAB was deleted from p2mutAB by digestion with AscI and BglII, with 1762bp of the full length 4101bp gene remaining. The fragment was blunt-ended with Klenow fragment (Roche) and re-ligated to create p2ΔmutAB. The ΔmutAB 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 (Fig. 5a). The remainder of the operonic genes and their corresponding ribosomal binding site (RBS) were intact. The PAg85-lacZ, Phsp60-sacB cassette marker gene cassette from pGOAL17 (Parish and Stoker, 2000) was inserted into the PacI sites of p2NIL to complete construction of the suicide delivery vector, p2ΔmutAB17 (Fig.S. 1.i). The mutAB deletion mutant of MTB H37Rv, ΔmutAB, was constructed by two-step allelic exchange mutagenesis using previously described methods (Gordhan et al., 1996; Parish and Stoker, 2000).

Genetic reversion of the mutAB mutation in the ΔmutAB mutant strain to generate the ΔmutAB::mutAB derivative was carried out by knock-in allelic exchange mutagenesis using the suicide plasmid, p2mutAB17, which contains the full-length mutAB genes plus 1431 bp of 3’- and 2228 bp of 5’-flanking chromosomal sequence (Fig.S. 1.ii). This vector was constructed by cloning the lacZ-sacB cassette from pGOAL17 in the PacI site of p2mutAB. The ΔprpDC, ΔmutAB ΔprpDC and ΔmutAB::mutAB ΔprpDC mutants were constructed by deletion of the prpDC gene in the H37Rv, ΔmutAB and ΔmutAB::mutAB backgrounds, respectively, using the previously described suicide plasmid, pAU100 (Muñoz-Elias et al., 2006). The ΔprpDC mutant was complemented genetically by integration of the previously described complementation vector, pPRPDC (Muñoz-Elias et al., 2006), at the attB locus. The Δicl1::hyg mutant was constructed by deletion of icl1 in H37Rv, using the knockout vector pJM056-1, previously described by McKinney et al. (McKinney et al., 2000). The episomal vector harboring icl1, pICL1, was used for complementation of Δicl1::hyg (Muñoz-Elias and McKinney, 2005). The plasmids pAU100, pPRPDC, pJM056-1 and pICL1 were all obtained as a kind gift from Prof. J. McKinney (EPFL, Switzerland). All mutant strains were genotypically confirmed by Southern blot analysis (Fig. 5a and Fig.S. 2 ), as previously described (Gordhan et al., 1996).
MTB genomic DNA containing the Rv1998c gene and flanking sequences was obtained from the BAC library (BAC Rv175 spanning region 2238065 to 2310745) (Brosch et al., 1998) and digested with SphI to obtain a 3640bp template which was cloned into pGEM3Z(+)f (Promega) to form pGEMRv1998c. This vector was digested with BamHI and HindIII and the 3670bp fragment containing Rv1998c was cloned into p2NIL (Parish and Stoker, 2000) to form p2Rv1998c. An internal, 312-bp region of Rv1998c was deleted from p2Rv1998c by digestion with NarI, with 464bp of the full length 777bp gene remaining. The vector was religated to create p2ΔRv1998c and confirmed by PCR (see Table S. 2 for a description of the primers and amplicon properties). The P_{Ag85-lacZ}, P_{hsp60-sacB} cassette marker gene cassette from pGOAL17 (Parish and Stoker, 2000) was inserted into the PacI sites of p2NIL to complete construction of the suicide delivery vector, p2ΔRv1998c17.

### 3.3 Heterologous over-production of a His-tagged recombinant form of Rv1998c in E. coli

The Rv1998c ORF was amplified by PCR with the Expand High Fidelity PCR System (Roche Molecular Biochemicals) from genomic MTB DNA. To facilitate cloning, the PCR primers used to generate the ORF were designed to contain BamHI and HindIII, respectively, as described in Table S. 2. The PCR product and pQE30xa (Qiagen) vector were both restricted with these two enzymes, and the PCR product was cloned into pQE30xa for over-expression of Rv1998c with an N-terminal His-tag. The sequence of the insert of the resulting vector, pQE30xa::Rv1998c, was confirmed. Chemically competent E. coli cells (XL-1 Blue, Stratagene) were transformed with the plasmid and maintained in Luria-Broth (LB) with 0.2% glucose to efficiently block transcription of Rv1998c. Overproduction of the His-tagged recombinant form of Rv1998c was performed by growing cells at 30°C in LB to an OD_{600} of 0.6-0.8 and equilibrating at room temperature (~18°C) for 1 h. Following induction of gene expression with 0.05 mM isopropyl thio-β-D-galactoside (IPTG), 1.5-ml samples were taken from cultures stirred at ~18°C over a 2-h time course of subsequent growth. As a positive control, the MCL from E. coli was over-expressed with an N-terminal His-tag in parallel using the pQE30::prpB expression vector (Brock et al., 2001) that was kindly provided by Dr. M.
Brock (Hans-Knöll-Institut, Germany). The cells were pelleted and resuspended in 300µl of ice-cold phosphate buffered saline (PBS). The cells were disrupted by sonication and the sample centrifuged at 4°C (15000 x g for 5 min) to separate fractions. Sample aliquots (15 µl) were fractionated by SDS-PAGE (12% acrylamide) using PageRuler prestained protein ladder (Fermentas) as a protein size marker. Overproduction of soluble *E. coli* MCL (PrpB) (32kDa) from room temperature induction was confirmed by SDS-PAGE. In contrast, the His-tagged Rv1998c recombinant protein (30 kDa) was localised exclusively in the insoluble fraction (Fig. 8). Induction conditions were adjusted in subsequent experiments to optimise for the production of small amounts of recombinant Rv1998c protein in the soluble fraction. Fifty ml cultures were induced at ~18°C at a lower cell density (OD₆₀₀=0.4) with 0.01mM IPTG overnight in flasks with a large surface area for improved aeration, before harvesting by centrifugation and resuspension in 1ml of lysis / column buffer (50mM Tris-HCl pH7.4, 0.3M NaCl, 5mM imidazole). Chromotographic enrichment of the His-tagged proteins was performed using His-Bind resin (Novagen). Briefly, after disruption by sonication, 500µl of cell lysate was loaded onto Ni-nitrilotriacetic acid (Ni-NTA) agarose (Novagen) with a bed volume of 200ul. The column was washed with lysis/column buffer to remove unspecifically bound proteins. MTB Rv1998c and *E. coli* MCL (PrpB) were each eluted with 1ml of elution buffer (50mM Tris-HCl pH 7.4, 250mM imidazole). The eluant was supplemented immediately with 2mM dithiothreitol (DTT) to prevent oxidation of any sensitive cysteine residues. To concentrate proteins for analysis by SDS-PAGE, ice-cold trichloroacetic acid (TCA) protein precipitation was done on the 1ml eluant. The protein pellet was resuspended in 100 µl sample buffer (10 × concentrate).

### 3.4 Tetracycline-inducible expression of Rv1998c in MTB

The Rv1998c open reading frame (ORF) was amplified by PCR in an Eppendorf MasterCycler Gradient using High Fidelity Phusion Polymerase (New England Biolabs) using the primers detailed in Table S. 2. The amplicon, which contains the entire Rv1998c ORF and its native ribosome binding site (RBS) was cloned downstream of the tetracycline (Tet) regulated mycobacterial promoter, P₉₅₇tetO, carried on the episomal plasmid, pSE100 (Ehrt *et al.*, 2005) by insertion between the BamHI and SphI sites of the vector. The resulting
construct, pSE100::Rv1998c, was confirmed by DNA sequencing of the region spanning the upstream tet-promoter to several bases after the end of the Rv1998c ORF. pSE100::Rv1998c (Fig.S. 1.iii and Table S. 1) and was co-electroporated into wild type H37Rv together with the integrative vector, pMC1s, which carries the Tet repressor (TetR) under the control of a strong mycobacterial promoter and directs integration of the $P_{smyc-tetR}$ fusion at the $attB$ site of the chromosome. Recombinants were selected on 7H10 agar plates that contained 50µg/ml Hyg, 25µg/ml Km without anhydrotetracycline (AHTc) inducer. To assess the possible MCL function of Rv1998c in a background devoid of MCL function attributable to ICL (Gould et al., 2006), a construct driving Tet-regulated expression of Rv1998c was also introduced into the ICL-deficient mutant of H37Rv ($\Delta icl::hyg$). However, since the $\Delta icl::hyg$ mutant carries a hyg resistance marker which precluded the use of pSE100::Rv1998c, an alternative expression strategy was employed in which the integrative vector, pMC1m (Ehrt et al., 2005), which carries $tetR$ under the control of a mycobacterial promoter of intermediate strength was modified to carry both $tetR$ and $P_{myc1tetO}$. An SpeI (blunted with Klenow) / ClaI digest of pSE100::1998c was used to excise the 1306-bp fragment containing the $P_{myc1tetO}$:Rv1998c transcriptional fusion. This fragment was cloned into ClaI / PvuII digested pMC1m and the final construct, pMC1m::$P_{myc1tetO}$::Rv1998c (Fig.S. 1.iv and Table S. 1) was electroporated into the $\Delta icl::hyg$ mutant strain. Confirmation of the transformants harbouring the integrated and / or episomal plasmids was done by PCR (see Table S. 2 for a description of the primers and amplicon properties).

### 3.5 Preparation of MTB cell-free extracts

Bacteria were grown to late log phase (OD$_{600}$=0.7-1.0) in 7H9 broth or propionate-containing media. Twenty ml of cultures were induced with AHTc at a concentration of 100ng/ml for 20 h for maximal expression (Ehrt et al., 2005), and collected by centrifugation (1100 × g for 20 min). The pellet was washed three times with phosphate-buffered saline containing 0.05% Tween-80 (PBST) and resuspended in 2ml of assay buffer. Cells were disrupted by bead beating (Savant Fastprep FP120) with 0.1mm zirconia-silica beads (Bio101 lysing matrix B) for 3 × 60s, and cooled on ice between bursts. Extracts were clarified by centrifugation (15000 × g for 15 min) and the aqueous solution filtered through a 0.45µm PVDF membrane filter.
(Millex). Protein concentrations were determined using the Biorad Protein Assay based on the method of Bradford (Bradford, 1976). A standard curve was generated with bovine serum albumin (Sigma). Cell extracts were used immediately for enzymatic assay.

3.6 2-Methylcitrate lyase assays

Lyophilised *threo*-2-methylisocitrate, the racemic mixture of (2R,3S)- and (2S,3R)-2-methylisocitrate, received as a kind gift from Dr. M. Brock (Hans-Knöll-Institut, Germany) was dissolved in 0.5M NaOH and heated at 95°C for 20 min to saponify the lactone ring. Saponified 2-MIC was diluted into 1M KH$_2$PO$_4$ at pH 7.0, before use in assays. MCL activity was measured by following the lactate dehydrogenase (LDH)-mediated reduction of pyruvate to lactate with concomitant oxidation of NADH (Warren, 1970). The 1-ml reaction mixture contained 50mM KH$_2$PO$_4$ at pH 7.0, 5mM MgCl$_2$, 0.1mM NADH, 7U LDH (Roche), 2mM DTT and and cell free extract (5-100µg in 5-100µl). Reactions were preincubated for 5 min and started by addition of 2mM 2-MIC (effectively half the concentration of the biologically active isomer (2R,3S)-2-methylisocitrate (Darley, 2003)). 2-MIC-stimulated NADH oxidation was measured spectrophotometrically at 340nm using the standard extinction coefficient 6.22mM$^{-1}$ for a 1cm path length. Background was measured and subtracted by carrying out mock reactions without addition of 2-MIC. Calculations were done using the equation $A=\varepsilon c l$, where $A$ is the change in absorbanc/min, $\varepsilon$ is the molar extinction coefficient, $c$ is the change in concentration (moles/litre) and $l$ is the pathlength.

3.7 Gene expression analysis by real-time quantitative reverse transcription-PCR (qRT-PCR)

The level of expression of the *prpD* (Rv1130), *icl* (Rv0467) and *mutB* (Rv1493) genes in H37Rv cultured on 7H9, propionate with and without vitamin B$_{12}$ and valerate with vitamin B$_{12}$ was determined by qRT-PCR. Cultures were grown to mid-log phase (OD$_{600}$ = 0.4), bacteria were harvested and RNA extracted using TRIzol (Sigma), as previously described.
(Downing et al., 2004). The cell suspensions were transferred to tubes containing Lysing Matrix B (Q Biogene) and lysed in a ribolysrer (Savant Fastprep FP120) for three cycles (20 seconds at speed 6) with cooling on ice for 1 min between pulses. Samples were then centrifuged (15000 x g for 45 seconds), and the solution above the beads and cellular debris removed to a tube containing 300µl of chloroform/isoamyl alcohol (24:1 v/v) and PhaseLock gel (Heavy; Sigma). Samples were mixed by rapid inversion for 15 s and then periodically for 2 min before centrifugation (15000 x g for 5 min). The top aqueous layer was transferred to a fresh tube and an equal volume of isopropanol added and samples incubated overnight at 4°C. Precipitated nucleic acids were collected by centrifugation (15000 x g for 20 min at 4°C), and pellets washed with 70% ethanol and air dried. Crude RNA samples were treated with DNase I (Ambion) by incubation at 37°C for 60 min and purified further using an RNeasy kit (Qiagen) according to the manufacturer’s instructions. The concentration of RNA was estimated using A_{260}/A_{280} spectrophotometric readings and the quality of purified total RNA was assessed by gel electrophoresis (2% agarose; 0.1% SDS). Samples were then subjected to a final DNase I treatment using the Ambion DNA-free™ kit, according to manufacturer’s instructions. Synthesis of cDNA was carried out at 60°C for 30 min using 500 ng of RNA in a 20 µl reaction mixture containing 1× PCR buffer without MgCl₂ (Roche), 12.5 nM reverse primers, 4 mM MgCl₂, 0.8 mM dNTPs, 3% DMSO and 1 µl of Enhanced Avian Reverse Transcriptase (Sigma), in an Eppendorf MasterCycler. Control reactions containing no reverse transcriptase were established in parallel to assess levels of contaminating DNA. For qRT-PCR, 2 µl of cDNA was used for amplification with LightCycler FastStart DNA Master SYBR Green I kit in the Roche LightCycler (version 1.5). Absolute quantifications of transcript levels using standard curves were performed with the LightCycler software (version 4.0). Absolute numbers of transcript were normalised to the number of sigA transcripts in the same sample and the normalised data were compared with normalised transcript levels in the 7H9 control. The analysis was performed in triplicate biological samples, each in duplicate. Primers used were designed using the Primer3 design programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are detailed in Table S. 2. The primers used to determine sigA transcript levels for normalization were as described by Dawes et al (Dawes et al., 2003).
The paired $t$-test was used to assess statistical significance of pair-wise comparisons using GraphPad Prism software (http://www.graphpad.com/quickcalcs/ttest1.cfm).

Tet-regulated expression of Rv1998c under the control of the $P_{\text{mycl\,tetO}}$ promoter-operator was analyzed by semi-quantitative RT-PCR using the primers described in Table S. 2. cDNA synthesis was carried out as described above and 2µl of cDNA or genomic DNA standard was used for PCR amplification in an Eppendorf Mastercycler. Products were visualised on a 2% agarose gel.
4 RESULTS

4.1 Pathways for propionate metabolism in MTB deduced from bioinformatic analysis

Early evidence for odd-chain fatty acid metabolism in MTB stems from studies in which respiration on heptadecanoic acid (C\textsubscript{17}) was observed (Bloch and Segal, 1956). At the outset of this study, preliminary experiments that were aimed at assessing the ability of the H37Rv strain of MTB to grow on propionate revealed that propionate did indeed support growth of H37Rv as an odd-chain fatty acid carbon source (data not shown). Similar findings were independently observed and subsequently published by other groups (Chang et al., 2007; Muñoz-Elias and McKinney, 2005; Muñoz-Elias et al., 2006). However, at the time of these initial investigations, no propionate oxidation pathways in MTB had been functionally characterised, prompting a detailed bioinformatic analysis of potential propionate oxidation pathways in MTB to be undertaken based on its complete genome sequence (Cole et al., 1998). Using search terms and sequences selected according to information obtained from the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database (www.genome.jp/kegg/pathway.html), keyword and BLAST searches (http://genolist.pasteur.fr/TubercuList/) against the MTB genome (Cole et al., 1998) revealed the presence of two putative pathways for propionate metabolism, namely the methylmalonyl pathway and the methylcitrate cycle (Fig. 2).
Fig. 2 Predicted pathways of propionate metabolism in MTB
Catabolism of alternative carbon sources including odd- and branched-chain FA, branched-chain amino acids (BCAA), and cholesterol generates propionyl-CoA as a three-carbon (C_3) terminal product. Propionyl-CoA can be oxidised to succinate through either the methylcitrate and glyoxylate cycles or the methylmalonyl pathway. Methylcitrate cycle enzymes include methylcitrate synthase (MCS), MCD and MCL. Glyoxylate cycle enzymes are ICL1, which also provides the methylisocitrate lyase (MCL) activity in MTB (Gould et al., 2006; Muñoz-Elias et al., 2006), and MLS. Methylmalonyl pathway enzymes are propionyl-CoA carboxylase (PCC), methylmalonyl-CoA epimerase (MMCE) and methylmalonyl-CoA mutase (MCM).

4.2 Constituents of the methylmalonyl pathway

The first step of the methylmalonyl-CoA pathway is catalyzed by a propionyl-CoA carboxylase (PCC; Fig. 2). The PCC from *Streptomyces coelicolor* has been characterised in detail and shown to consist of three different components: a larger subunit (α chain) with the ability to carboxylate its covalently bound biotin group (AccA2), a smaller subunit (β chain) bearing the carboxyltransferase activity (PccB), and the third ε subunit essential for maximal activity of the complex (Pccε) (Diacovich et al., 2002; Rodriguez and Gramajo, 1999). Two separate reactions are catalysed by this enzyme:

\[
\text{HCO}_3^- + \text{Mg-ATP} + \text{enzyme-biotin} \rightleftharpoons \text{enzyme-biotin-CO}_2^- + \text{Mg-ADP} + \text{Pi}
\]

\[
\text{Enzyme-biotin-CO}_2^- + \text{propionyl-CoA} \rightleftharpoons \text{enzyme-biotin} + \text{methylmalonyl-CoA}
\]

Several complexes have been purified from a number of other actinomycetes (Erfle, 1973; Haase et al., 1982; Hunaiti and Kolattukudy, 1982). These complexes are referred to as acyl-CoA carboxylases (ACC) as they are able to carboxylate acetyl- propionyl-, or butyryl-CoA. The MTB genome contains several genes encoding either the α (*accA1, accA2* and *accA3*) or β (*accD1-6*) subunits, which together form at least three distinct multi-functional ACC complexes. These presumably serve distinct biological roles in providing malonyl-, methylmalonyl-, or ethylmalonyl-CoA extender units for the biosynthesis of different polyketides and fatty acids (Cole *et al.*, 1998). Of the six β subunits encoded by MTB, BLAST analysis identified AccD5 as having the highest homology (65% sequence identity) to the β subunit of the characterised PCC complex of *S. coelicolor* (PccB). However, the aspartic
residue (D422) believed to be responsible for substrate selectivity in PccB is replaced by a cysteine (C437) in the AccD5 of MTB (Diacovich et al., 2004). However Gago et al. found that replacing the aspartic residue of PccB with a cysteine did not alter specificity for propionyl-CoA (Gago et al., 2006). The protein sequences of the carboxytransferases found in MTB and other actinomycetes were analysed using ClustalW (Version 2; www.ebi.ac.uk/Tools/) and used to construct a phylogenetic tree (Fig. 3).

Fig. 3 Phylogenetic analysis of β subunits of ACCase complexes found in actinomycetes
The phylogenetic tree was generated using Clustal W2. The organism abbreviations are MTB, M. tuberculosis; Pa, Propionibacterium acnes; Sc, S. coelicolor; Cg, C. glutamicum.

Consistent with the clustering of AccD5 with PCCase from other actinomycetes, the PCCase complex in MTB, which was biochemically characterised by others during the course of this study, was found to comprise α, β and ε subunits encoded by accA3 (Rv3285) and accD5 (Rv3280) cotranscribed with accE5 (Rv3281), respectively (Gago et al., 2006; Oh et al., 2006).

PCC stereospecifically synthesises (S)-methylmalonyl-CoA. Either the (R) or (S) enantiomer of methylmalonyl-CoA can be used as the chain extending substrate by mycoserosic acid synthase-like enzymes for multimethyl-branched fatty acid biosynthesis (Rainwater and Kolattukudy, 1985). This is contrary to the MCM which recognises only the R-stereoisomer of methylmalonyl-CoA mutase (Retey and Lynen, 1964), as the methyl group of the S-isomer would sterically clash with Tyr-89 (see below), thus excluding it from the active site (Banerjee and Vlasie, 2002). This diversion is rectified by methylmalonyl-CoA epimerase (MMCE) which catalyses the conversion of the (S)-methylmalonyl-CoA to the (R)-methylmalonyl-CoA.
configuration, keeping the methylmalonyl-CoA enantiomers in isotopic equilibrium (Reszko et al., 2003). Based on BLAST analysis, Rv1332A was assigned as the putative MMCE in MTB with approximately 60% similarity to characterised MMCE from other bacteria (Bobik and Rasche, 2004; Leadlay, 1981).

The MTB genome was analyzed further for a MCM responsible for the catalysis of the carbon skeleton rearrangement of (R)-methylmalonyl-CoA to succinyl-CoA in a vitamin B\textsubscript{12} dependent manner (Fig. 2). MTB contains orthologues of the MCM genes (Cole et al., 1998; Fleischmann et al., 2002), with Rv1492 (mutA) and Rv1493 (mutB) annotated as the β (65kDa) and α (80kDa) subunits, respectively, of the MCM heterodimer. Cluster analysis grouped the MTB α subunit with mutB from P. shermanii (Fig. 4). These branched separately from the MeaA from S. cinnamonensis, a B\textsubscript{12}-dependent MCM-like protein (Smith et al., 1996; Zhang and Reynolds, 2001) and the homodimeric MCM, sleeping beauty mutase (Sbm) from E. coli (Fig. 4) (Froese et al., 2008; Haller et al., 2000).

**Fig. 4 Phylogram of MutB orthologues**
The phylogram was generated by ClustaW2 and shows clustering of Methylobacterium extorquens AM1 (ME) MeaA; Genbank accession number AAC44087, Streptomyces cinnamonensis (SC) MeaA; Genbank accession number AAG40840, M. tuberculosis (MTB) MutB; Genbank accession number CAB02043, Propionibacterium shermanii (PS) MutB; Genbank accession number P11653, E. coli (EC) Sbm; Genbank accession number P27253 and Streptomyces cinnamonensis (SC) MutB; Genbank accession number Q05065.

Key prerequisites including putative RBS sequences and lack of stop codon interruptions within the ORFs of interest were noted. Each ORF was further analysed for the conservation of signature motifs and important amino acids. Only the larger α subunit has a C-terminal coenzyme B\textsubscript{12} binding domain characterised by the presence of the conserved DXHXXG motif (Marsh and Holloway, 1992), and presumably binds a single molecule of cofactor per
heterodimer. The entire constellation of conserved active site residues, Y243 (Y259), H244 (H260), Y89 (Y105) and R207 (R223) are present in the α subunit (using the MCM from P. shermanii as reference numbering (Banerjee and Vlasie, 2002) with the corresponding MTB numbering shown in brackets). These active site residues form an aromatic corridor for substrate placement and play critical roles in controlling radical reactivity and protect against inactivating side reactions such as electron transfer from cob(II)alamin to the substrate leading to hydroxycobalamin (OHCbl) formation (Thoma et al., 2000; Vlasie and Banerjee, 2004). Further stabilization by an auxiliary protein which complexes to MCM, known as MeaB, is thought to protect against this suicidal inactivation during the course of turnover (Korotkova and Lidstrom, 2004; Padovani and Banerjee, 2006), promoting an extended enzyme half-life (~170 min for P. shermanii MCM; (Thoma et al., 2000)). Rv1496 was assigned as the putative meaB orthologue, showing high homology (40-70% similarity) to meaB from other organisms.

Notably, the MTB genome encodes three B₁₂-dependent enzymes (Cole et al., 1998), and while polymorphisms in the ribonucleotide reductase (Dawes et al., 2003; Tsolaki et al., 2004) and the methionine synthase (Fleischmann et al., 2002; Warner et al., 2007) have been reported in clinical strains, the MCM is highly conserved. Importantly, close inspection of this locus in clinical strains F11, CDC1551, Haarlem and C clinical strains showed no allelic variation.

Interestingly, bioinformatic analysis of the mutAB operon revealed a predicted mazEF-type toxin-antitoxin (TA) module encoded by Rv1494 and Rv1495, respectively, embedded between mutAB and the putative meaB. Toxin-antitoxin (TA) systems were originally found associated with low copy number plasmids, increasing the effective stability by selectively eliminating plasmid-free progeny (post segregational killing; (Yarmolinsky, 1995)). The molecular basis underlying this ‘addiction’ is based on the rapid degradation of an unstable antitoxin protein, releasing stable toxin from the TA complex to exert its lethal activity in plasmid free daughter cells. In this way maintenance of the plasmid is ensured. Although their function is the subject of intense interest and debate, it has been proposed that chromosomally encoded TA modules may function in genome stabilization by limiting the loss of flanking DNA (Szekeres et al., 2007). Therefore, it is conceivable that the mazEF-type TA module
may stabilise the \textit{mutAB-meaB} locus through a TA-dependent mechanism (Szekeres \textit{et al.}, 2007).

\textbf{4.3 Vitamin B$_{12}$ supplementation enables a functional methylmalonyl pathway}

Since it appeared that MTB possessed the complete complement of genes for the methylmalonyl pathway, the \textit{mutAB} gene predicted in the bioinformatics search to form part of the methylmalonyl pathway in H37Rv was in part deleted by allelic exchange mutagenesis (Fig. 5a) and the mutant strain assessed for propionate utilisation. Surprisingly, growth of $\Delta$\textit{mutAB} on 0.1% propionate was indistinguishable from wild type (Fig. 5b.i). These observations established that the methylmalonyl pathway was not essential for growth on 0.1% propionate. Furthermore these results indicate the functionality of the alternate propionate oxidizing pathway in MTB, the methylcitrate cycle.

During work probing the functionality of the B$_{12}$-dependent methionine synthase, MetH, in MTB, it was discovered that it was necessary to supplement growth media with vitamin B$_{12}$ to facilitate operation of this enzyme (Warner \textit{et al.}, 2007). Since MCM also requires a vitamin B$_{12}$-derived coenzyme for functionality, the propionate-containing culture medium was supplemented with vitamin B$_{12}$ to ensure that this coenzyme was not deficient. Growth on 0.1% propionate was enhanced in the presence of vitamin B$_{12}$ supplement (Fig. 5b.ii). This suggested that MTB may not synthesise the coenzyme necessary for MCM activity under these conditions. Since MTB possesses two other vitamin B$_{12}$-dependent enzymes, it was important to determine if the growth advantage afforded by vitamin B$_{12}$ supplementation was specifically \textit{mutAB}-dependent. $\Delta$\textit{mutAB} was grown in 0.1% propionate media supplemented with vitamin B$_{12}$ (Fig. 5b.ii). Growth of $\Delta$\textit{mutAB} with vitamin B$_{12}$ was similar to H37Rv without vitamin B$_{12}$. This indicates that this growth advantage is mediated specifically by MCM (Fig. 5b.ii) and that exogenous vitamin B$_{12}$ exerts no observable pleiotropic effects on propionate metabolism. As growth of $\Delta$\textit{mutAB} was not abolished under conditions of vitamin B$_{12}$ supplement, it was evident that vitamin B$_{12}$ did not negatively regulate the alternative propionate metabolic pathway. Since the methylcitrate pathway appears not to be negatively regulated by vitamin B$_{12}$ the activity of the methylmalonyl pathway is unlikely to be soley
responsible for the improved growth on vitamin B\textsubscript{12}-supplemented propionate. The growth of the complemented \( \Delta \text{mutAB} \) mutant in the presence of vitamin B\textsubscript{12} was similar to that of wild type with vitamin B\textsubscript{12} (data not shown).

It was considered that higher concentrations of propionate may demand the synergistic operation of two or more propionate oxidizing pathways. Therefore, 0.2\% propionate was subsequently used to further dissect out any possible contribution to growth by the methylmalonyl pathway. Growth of \( \Delta \text{mutAB} \) was retarded relative to H37Rv (Fig. 5b.iii). This observation was contingent on vitamin B\textsubscript{12} supplementation as growth of H37Rv on 0.2\% propionate without vitamin B\textsubscript{12} supplement is similar to \( \Delta \text{mutAB} \) (results not shown).

Therefore, in summary, the observations displayed in Fig. 5 established the following: (i) MTB does not synthesise the necessary coenzyme required for MCM activity \textit{in vitro} under the conditions tested; (ii) the growth stimulatory effect of vitamin B\textsubscript{12} is strictly dependent on, and mediated by \textit{mutAB} as improved growth is restored in the reversion mutant, \( \Delta \text{mutAB}::\text{mutAB} \); (iii) the methylmalonyl pathway enzymes, namely PCC, MMCE and vitamin B\textsubscript{12}-dependent MCM are functional, and; (iv) more than one propionate oxidizing pathway may be required for optimal growth of MTB on 0.1 and 0.2\% propionate.
Fig. 5 Construction and characterisation of the ΔmutAB mutant of MTB H37Rv

a. Construction and genotypic characterization of ΔmutAB and its reverted (knock-in) derivative (ΔmutAB::mutAB)

The mutAB and flanking genes are shown as solid arrows (not to scale). The line drawing above shows the 2342-bp segment internal to mutAB that was deleted in the ΔmutAB mutant strain (hatched box). For the Southern blot analysis (right), genomic DNA from the wild type, ΔmutAB and reverted (ΔmutAB::mutAB) strains was digested with SphI which cuts on either side of the deleted region to produce a 5870-bp fragment from H37Rv and ΔmutAB::mutAB and a 3528-bp fragment from ΔmutAB which were detected using the PCR-generated probe denoted by a grey box.

b. Growth of ΔmutAB on propionate

(i) Growth of H37Rv (■) and ΔmutAB (◆) on 0.1% propionate.
(ii) Improved growth of MTB on 0.1% propionate supplemented with vitamin B₁₂ is mutAB-dependent. H37Rv with (□) and without (■) vitamin B₁₂; ΔmutAB with (◇) and without (◆) vitamin B₁₂.
(iii) Improved growth on 0.2% propionate supplemented with vitamin B₁₂ is mutAB-dependent. (◇), ΔmutAB; (▲), ΔmutAB containing the reverted mutAB allele (ΔmutAB::mutAB); (■), H37Rv.
4.4 Constituents of the methylcitrate cycle

The MTB genome encodes homologues of MCS (prpC; Rv1131) and MCD (prpD; Rv1130) which are part of the same operon (Fig. 2). Significantly, no MCL-encoding homologue (prpB) was immediately evident, despite the presence of prpB homologues in other mycobacteria in an operonic arrangement with prpD and prpC (Claes et al., 2002; Upton and McKinney, 2007). This suggested that a non-orthologous enzyme may have taken over the role of the classical MCL (PrpB) enzyme in MTB. The MCL (PrpB) enzyme catalyses the cleavage of 2-methylisocitrate to succinate and pyruvate, analogous to the reaction catalysed by ICL in the glyoxylate cycle (Fig. 2). Although, E. coli ICL has no activity on 2-methylisocitrate (Liu et al., 2005), some activity for 2-methylisocitrate, albeit 5-50-fold lower than the activity for the cognate substrate, isocitrate, has been reported for the ICL enzymes from Neurospora crassa, Pseudomonas indigofera, Chlorella vulgaris (McFadden et al., 1972) and Saccharomyces cerevisae (Luttik et al., 2000). At the time of this bioinformatic analysis, it had been proposed that the substrate selectivity of these two enzymes could be attributed to steric restrictions: the methyl group (C$_2$-CH$_3$) present in pyruvate, but not in glyoxylate, could be accommodated by a hydrophobic depression in PrpB formed by F186, L234 and P236 (using the E. coli PrpB numbering) that is not present in ICL (Grimm et al., 2003). W283, F345 and T347 are the equivalent conserved residues in ICL1 of MTB. In MTB, modeling predicted that addition of the methyl group to the glyoxylate bound in the ICL enzyme would form short van der Waals contact with T347 thereby precluding 2-methylisocitrate from being accommodated in the hydrophobic pocket of ICL (Grimm et al., 2003; Liu et al., 2005; Simanshu et al., 2003). A T347A substitution in ICL1 (reducing the size of the side chain) was proposed as a way to achieve reversal of substrate specificity (Liu et al., 2005). However, since all characterised ICLs have the identical binding pocket triad to MTB ICL1 (Grimm et al., 2003; Liu et al., 2005), these predictions were inconsistent with reported activity of ICLs on 2-methylisocitrate, as mentioned above. Therefore, ICL could not be absolutely excluded as a candidate PrpB in MTB. Importantly, however, the ICLs with minimal MCL activity from Aspergillus nidulans and S. typhimurium are unable to support growth of these organisms on propionate as a sole carbon source (Brock, 2005; Horswill and Escalante-Semerena, 1999; Pronk et al., 1994). Further differences in the enzyme active site
may account for the minimal MCL activity observed in these ICLs. It has been proposed that for substrate binding and catalysis a drastic conformational change of a loop spanning 10-11 amino acid residues containing the signature sequence KKCGH in all ICLs and KRCGH in all MCLs, including the active cysteine, is necessary. Importantly, mutation of KKCGH to KRCGH in the ICL from *E. coli* led to an 89% loss of activity (Rehman and McFadden, 1997). Overall, the evidence suggested very little overlap in function, with MCLs possessing no ICL activity (Brock *et al.*, 2001; Grimek *et al.*, 2003; Liu *et al.*, 2005) and ICLs possessing minimal MCL activity. This provided the rationale for postulating that an enzyme other than ICL may perform the MCL function necessary for growth of MTB on propionate.

### 4.4.1 Identification of Rv1998c as a putative MCL

Bioinformatic analysis identified a single MCL candidate in the MTB proteome, namely, the conserved hypothetical protein (CHP), Rv1998c, which belongs to the Cluster of Orthologous Groups (COG) 2513 (Tatusov *et al.*, 1997). Members of COG 2513 are part of the $(\alpha/\beta)_8$ ICL/PEPM enzyme superfamily and include, amongst others, phosphoenolpyruvate mutase (PEPM) and MCL.

PEPMs are involved in the formation of C-P bonds in the biosynthesis of phosphonate secondary metabolites (Hidaka *et al.*, 1990; Pollack *et al.*, 1992). Interestingly, MCL shares greater sequence similarity with PEPM enzymes (30-37%) than with ICL (23-32%) (Jia *et al.*, 1999). Structure-function analyses have shown that the mutases catalyse very different reactions to the lyases (Britton *et al.*, 2001; Liu *et al.*, 2002; Sharma *et al.*, 2000), but despite this divergent chemistry, the same catalytic scaffold is conserved (Huang *et al.*, 1999; Liu *et al.*, 2004), which accounts for high structural similarity/conservation. The core residues characteristic of the ICL/PEPM family members are conserved as they bind the common substrate $\alpha$-C(=O)COO unit and the Mg$^{2+}$ cofactor (Lu *et al.*, 2005) (Fig. 6). The core chemistry involves the stabilization of an oxyanion intermediate.
Fig. 6 Amino acid alignment of representatives of the ICL/PEPM superfamily

Multiple sequence alignment using Clustal Version 2.0.5. PrpB from *E. coli*, CPEP (carboxyPEP mutase) from *Streptomyces hygroscopicus*, PDP (petal death protein) from *D. caryophyllus*, PEPM from *Mytilus edulis* and Rv1998c from MTB. The core residues are highlighted in yellow and the key diversification residues (glutamic acid and cysteine) shown to be essential for isocitrate and methylisocitrate lyase activity are denoted in red italics.
Overall this family of proteins comprises an evolutionarily diverse group of enzymes, which include oxaloacetate acetylhydrolase (OXAHYD), phosphonopyruvate hydrolases (PPYRH), and ketopantonoate hydroxymethyl transferase (PANB). These all catalyze different reactions by binding substrate differently inside the barrel (Liu et al., 2005). This diverse functional specialization from the members of this family may lead to different undiscovered functions for the remaining CHPs which fall within this superfamily.

Consistent with inclusion of Rv1998c in the ICL/PEPM family, Modbase (http://modbase.compbio.ucsf.edu/), a three-dimensional protein structure database, threaded the Rv1998c protein sequence most closely with two other members of this family, namely PrpB of E. coli Template Protein Data Bank (PDB) code: 1mumA, 21% sequence identity (Psi-Blast e-value 8e-36); and with another protein also having MCL activity (Lu et al., 2005; Teplyakov et al., 2005) called petal death protein (PDP) of Dianthus caryophyllus PDB code: 1zlpA, 22% sequence identity (Psi-Blast e-value 4e-27). In addition, homology modeling of Rv1998c was carried out by the Robetta server (http://www.robetta.org/), using E. coli PrpB (Fig. 7).
Fig. 7 Model of Rv1998c fitted to PrpB of *E. coli* 
Superimposition of *in silico*-modeled Rv1998c (blue) and the X-ray crystal structure of *E. coli* PrpB (turquoise). Spdb viewer was used to generate the superimposed image. Rv1998c is merged with PrpB where the trace of the polypeptide chain is similar. The open conformation of the active site loop which is the conserved catalytic scaffold regulating solvent access in the apo structure is marked in black and contains the lyase signature motif KRC*GH (absent from Rv1998c) highlighted in red. In Rv1998c, this active site capping loop is truncated. The amino acids (using *E. coli* PrpB numbering) around the active residue C123 of the KRC*GH motif and the catalytic E188 residue are drawn with the side chains in red. The amino acid residues F186, L234 and P236 that form the hydrophobic pocket are colored in green. Mg$^{2+}$ is shown as a pink sphere.
Notably, a cysteinyl residue that is the active site residue for the C-C lyases, surrounded by the scaffold residues, KRCGH, which form the active site gating loop, is not conserved in the PEPMs (Grimek et al., 2003; Nimmo et al., 1989) and is similarly absent in Rv1998c (Fig. 6 and Fig. 7). However, since Rv1998c was the only candidate enzyme identified as part of COG 2513 in MTB, the possibility that Rv1998c has residual MCL activity by virtue of a novel nonorthologous catalytic mechanism, was investigated.

4.4.2 Analysis of the MCL function of Rv1998c

4.4.2.1 Over-expression of Rv1998c in E. coli

Recombinant His-tagged Rv1998c was over-expressed in E. coli in order to assess whether this protein possesses detectable MCL activity. Unlike the recombinant E. coli PrpB control, which was highly soluble, the His-tagged Rv1998c recombinant was found to be highly insoluble under the conditions tested (Fig. 8).
Fig. 8 Expression of recombinant Rv1998c in E. coli
a. Schematic representation of expression vector pQE30xa::Rv1998c
Rv1998c was fused in-frame to an N-terminal His-tag under the control of the lac operator.

b. SDS-PAGE analysis of expression of recombinant, His-tagged forms of MTB Rv1998c and E. coli PrpB in E. coli
Samples taken 30, 60 and 120 min after IPTG induction were fractionated by SDS-PAGE on 12% polyacrylamide gel. The soluble and insoluble fractions from the various samples were loaded on separate gels for analysis of recombinant forms of PrpB from E. coli and Rv1998c from MTB.

Conditions were therefore varied by changing the temperature and length of induction (refer to section 3.3) in order to identify conditions that would allow the expression of a soluble form of recombinant His-tagged Rv1998c in E. coli. To detect possible trace amounts of recombinant Rv1998c in the soluble fraction, this fraction was concentrated by passage through a Ni-Sepharose column followed by a TCA precipitation. The protein pellet was resuspended in sample buffer and assessed by SDS-PAGE (Fig. 9). However, no protein band corresponding to His-tagged Rv1998c was visible. In contrast, as reported by Brock et al., soluble recombinant PrpB from E. coli was readily detected as a band migrating at 32kDa in the soluble fraction of the E. coli host strain harboring the pQE30::prpB expression vector (Brock et al., 2001).
Fig. 9  SDS-PAGE of soluble cell extract after affinity chromatography on Ni-Sepharose column
Aliquots of 5, 15 or 20 µl were run on 12% SDS-PAGE after affinity chromatography and TCA precipitation of soluble cell extract (10× concentrated). Vector only (pQE30xa) and soluble *E. coli* PrpB were run as negative and positive controls, respectively.

4.4.2.2 Induction of Rv1998c expression in MTB and MCL assay of MTB cell extracts.

Given the insolubility of this recombinant protein in *E. coli*, possible MCL activity of Rv1998c in its natural host was investigated. Semi-quantitative RT-PCR was used to assess the expression of Rv1998c in MTB cultured under standard aerobic growth conditions in 7H9 media (Table 1, column 4). However, no Rv1998c transcript was detected under these conditions. This gene has been identified as a member of the DosR regulon (Voskuil *et al.*, 2003) which is induced by hypoxia and treatment with low-dose NO (Shi *et al.*, 2005). This gene was thus conditionally expressed by placing it under the control of a Tet-inducible
promoter (Ehrt et al., 2005) as described in section 3.4. To facilitate detection of MCL contributions from Rv1998c, MTB harbouring the recombinant construct was grown in 7H9 medium to an OD$_{600}$ of 0.8-1.0, where levels of ICL1 are low ((Wayne and Lin, 1982); Anna Upton, personal communication) and induced for Rv1998c expression.

During the course of this study, biochemical and structural evidence that ICL1 plays a dual role in the glyoxylate and methylcitrate cycles was published by Gould et al. (2006) who reported some recovery of MCL activity from recombinant ICL1 ($k_{cat} / K_m$ of $1.74 \times 10^3$ M$^{-1}$ s$^{-1}$; a measure of catalytic efficiency), although notably 367 fold less than E. coli PrpB , 3678 fold less than A. nidulans PrpB (using the pure enantiomer of MIC) (Brock et al., 2001) and 2413 fold lower than S. enterica PrpB (Grimek et al., 2003). The crystal structure of ICL1 revealed that the active site could indeed accommodate the additional methyl group of 2-methylisocitrate. Clearly, the substrate specificity of ICL1 does not arise from a single determinant, consistent with other findings which suggest that the size of the hydrophobic pocket depends to a large extent on the backbone placement of the triad residues (Teplyakov et al., 2005). Therefore to eliminate any MCL contribution from ICL1, an icl1 mutant of H37Rv was constructed using a knock-out vector obtained as a kind gift from Prof. J. McKinney (EPFL, Switzerland). The Δicl1 mutant was transformed with the vector carrying R1998c under control of the Tet-regulated promoter and induced similarly. Growth on propionate of the Δicl1 mutant overexpressing Rv1998c was not assessed due to the absence of ICL1 activity necessary to support the function of the glyoxylate cycle which may be required in conjunction with the methylcitrate cycle for growth on this carbon source (Textor et al., 1997; Wang et al., 2003; Wegener et al., 1969).

Induction of Rv1998c expression was monitored by detecting Rv1998c transcript levels by semi-quantitative PCR (Table 1). Rv1998c was successfully over-expressed in a AHTc-dependent manner in both wild type H37Rv and the Δicl1 mutant. MCL activity assays were performed using crude cell extract (Table 1). MTB grown on propionate (which has been shown to induce ICL1; (Muñoz-Elias et al., 2006)) was used as a positive control for MCL activity. The results from one representative experiment are shown in Table 1 using the assay conditions described under in section 3.6 (Brock et al., 2001; Muñoz-Elias et al., 2006). The
MCL activity for H37Rv grown in propionate (positive control) was comparable with that observed by Muñoz-Elías et al. (2006) and served as a positive control.

**Fig. 10 Schematic of construct used for Tet-regulated expression of Rv1998c in MTB**

Using the episomal plasmid, pSE100 (Ehrt et al., 2005) the region upstream of the start codon of Rv1998c is replaced with Pmyc1tetO.

<table>
<thead>
<tr>
<th></th>
<th>H37Rv::Pmyc1tetO1998c::pMC1s</th>
<th>H37Rv::Pmyc1tetO1998c::pMC1s</th>
<th>Δicl1::hyg::Pmyc1tetO1998c::pMC1m</th>
<th>H37Rv::pSE100 with pMC1s</th>
<th>H37Rv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>7H9</td>
<td>7H9</td>
<td>7H9</td>
<td>7H9</td>
<td>0.1 % propionate</td>
</tr>
<tr>
<td>AHTc</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>Rv1998c</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>sigA</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>MCL activity *</td>
<td>2.2</td>
<td>3.4</td>
<td>&lt; 2</td>
<td>2.6</td>
<td>27.2</td>
</tr>
</tbody>
</table>

**Table 1 Conditional expression of Rv1998c in MTB**

Semi-quantitative RT for Rv1998c and sigA was done on strains harboring episomal Rv1998c under the control of Pmyc1tetO. Column1, H37Rv::Pmyc1tetO1998c with pMC1s induced; Column2, H37Rv::Pmyc1tetO1998c::pMC1s uninduced; Column3, Δicl1::hyg::Pmyc1tetO1998c::pMC1m induced; Column4, H37Rv::pSE100::pMC1s induced. Control reactions without reverse transcriptase revealed no contaminating DNA (not shown). MCL enzyme activity in cell free extracts of MTB: *Units are defined as nanomoles of 2-methylisocitrate-stimulated NADH consumption min$^{-1}$ (mg protein)$^{-1}$ in the cell free extract. The detection limit was 2 nmol min$^{-1}$ mg$^{-1}$. Representative data from one experiment of two are shown.
No increase in MCL activity was detected in cell extracts from any of the cultures in which Tet-induced Rv1998c transcript was detected. Further, a complete loss of detectable MCL activity was found in cell extracts induced for Rv1998c, but lacking ICL1. This experiment was repeated with cultures induced for 6 h to minimise the potential toxicity of high protein expression levels, but similar findings were obtained (data not shown). (Muñoz-Elias et al., 2006). The absence of MCL activity in the ∆icl1 strain of H37Rv grown in 7H9 is consistent with the undetectable levels of MCL activity subsequently reported for the ∆icl1 ∆icl2 strain of Erdman grown in 0.2% glucose (Muñoz-Elias et al., 2006). Together, these results indicated that Rv1998c does not provide detectable MCL activity in MTB under the conditions tested.

### 4.4.3 Deletion of Rv1998c in the ∆icl1 mutant of MTB Erdman

In terms of ICL function, an important difference exists between the H37Rv and Erdman strains of MTB. Erdman has two distinct ICL-encoding genes, icl1 and icl2 (Honer Zu Bentrup et al., 1999). The icl2 (or aceA) gene encodes a functional ICL enzyme, although no MCL activity was detected for this protein ($k_{cat} < 1 \times 10^{-3} \text{s}^{-1}$). In H37Rv and other strains belonging to the Euro-American lineage, the aceA gene is split into two open reading frames, aceAa (Rv1915) and aceAb (Rv1916) which precludes the formation of ICL2 in H37Rv either as a single protein or through the association of the separate aceAa and aceAb modules. Consequently, in H37Rv, both ICL and MCL activity is provided exclusively by ICL1.

Despite the evidence that ICL1 provides MCL activity in MTB, an icl1 mutant of the Erdman strain was still able to grow (albeit poorly) on propionate (Muñoz-Elias and McKinney, 2005). Since recombinant ICL2 possesses no detectable MCL activity, the poor growth of this mutant on propionate (Lin et al., 2008; Muñoz-Elias and McKinney, 2005), combined with the residual MCL activity detected in cell extracts of this strain (2.62 ± 0.45 units; (Muñoz-Elias et al., 2006)) and the 190-fold lower MCL activity in ∆icl1∆icl2 complemented with ICL2
(4.03 ± 0.90 units) as compared to ICL1 (771.36 ± 83.00 units) (Muñoz-Elias et al., 2006) supports the existence of another enzyme with MCL activity in this organism.

To determine whether Rv1998c was accountable for growth of Δicl1 Erdman MTB in propionate, a knock-out vector for Rv1998c was constructed (section 3.2) and electroporated into the Δicl1 Erdman strain. Several attempts were made to obtain single cross-over homologous recombinants in the icl1 mutant of this Erdman strain although these were unsuccessful. However, parallel electroporations into the MTB H37Rv strain alternately resulted in several transformants. However, owing to time constraints, these experiments were suspended.

4.5 Growth of MTB H37Rv on propionate is attributable to the methylcitrate cycle

During the course of this study, evidence for the functionality of the methylcitrate cycle in the Erdman strain of MTB was reported (Muñoz-Elias et al., 2006). To assess the functionality of this pathway in MTB H37Rv, and to establish its role in supporting the growth of this strain on propionate, a mutant of H37Rv with the prpDC genes deleted was generated using the same suicide plasmid as was used to create a ΔprpDC mutant of the Erdman strain of MTB (Muñoz-Elias et al., 2006). As shown in Fig. 11, the prpDC mutant of H37Rv could not grow on propionate whereas its complemented counterpart grew as well as the wild type. These results confirmed the essentiality of the methylcitrate cycle for growth of H37Rv on this carbon source, as established previously by Muñoz-Elías et al. for the Erdman strain (Muñoz-Elias et al., 2006).
Fig. 11 The methylcitrate cycle is required for growth of H37Rv on propionate
The effect of loss of prpDC function on growth of H37Rv. ◆, H37Rv; □, ΔprpDC; ▲, complemented ΔprpDC mutant (ΔprpDC::prpDC).

4.6 Vitamin B$_{12}$ supplementation enables growth of a prpDC mutant

The results of section 4.3 established that supplementation of propionate-containing medium with vitamin B$_{12}$ enabled the activity of MCM, thereby facilitating growth of MTB on this carbon source. The ability of the methylmalonyl pathway to support growth of ΔprpDC on propionate when the media was supplemented with vitamin B$_{12}$ was therefore investigated. The results shown in Fig. 12 demonstrate that the ΔprpDC mutant can indeed grow in vitamin B$_{12}$-supplemented propionate media. Therefore, the methylcitrate cycle is conditionally essential in the absence of vitamin B$_{12}$ supplement. This finding reinforces the operation of the methylmalonyl pathway subject to the availability of vitamin B$_{12}$, consistent with our earlier observations of improved growth on vitamin B$_{12}$ supplemented propionate (Fig. 5b. ii and iii). These findings also contribute to validating the existence of a pathway for
cyanocobalamin uptake into MTB and conversion to the active adenosylcobalamin form by a functional ATR.

To confirm that growth on propionate supplemented by vitamin B$_{12}$ was mediated solely by MCM, a $\Delta$mutAB $\Delta$prpDC double mutant was made. This mutant was unable to metabolise propionate as indicated by its inability to grow on this carbon source (Fig. 13). However, reversion of the $\Delta$mutAB mutation to mutAB$^+$ in this mutant background ($\Delta$mutAB::mutAB $\Delta$prpDC) restored growth in the presence of vitamin B$_{12}$ (Fig. 13) showing unequivocally that mutAB encodes a functional apoenzyme which must be reconstituted with adenosylcobalamin obtained from cyanocobalamin supplement. However, the reversion mutant did not grow as well as the $\Delta$prpDC comparator strain (Fig. 13). 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.

Growth dependent only on the methylmalonyl pathway ($\Delta$prpDC with vitamin B$_{12}$; Fig. 12) was notably poorer than growth dependent only on the methylcitrate cycle (H37Rv without vitamin B$_{12}$; Fig. 11). As addition of vitamin B$_{12}$ did not enable the equivalent growth of $\Delta$prpDC (Fig. 12) to that of wild type growth without vitamin B$_{12}$ (Fig. 11), the effect of increasing concentrations of vitamin B$_{12}$ supplement on growth was assessed. Vitamin B$_{12}$ was titrated from 1 to 20µg/ml and found to be saturating for growth at 7.5µg/ml (Fig. 14). Therefore, the observed growth kinetics of $\Delta$prpDC in propionate supplemented with 10µg/ml vitamin B$_{12}$ (Fig. 12) established that supplementation with vitamin B$_{12}$ was not growth limiting.
Fig. 12 Growth on propionate supplemented with 10 µg/ml vitamin B$_{12}$.  
◆, H37Rv; □, ΔprpDC; ▲, complemented ΔprpDC mutant (ΔprpDC::prpDC).

Fig. 13 Vitamin B$_{12}$ supplementation enables mutAB-dependent growth of a prpDC-deficient mutant of H37Rv on propionate  
■, ΔprpDC; ◊, ΔmutAB ΔprpDC; ▲, ΔmutAB ΔprpDC double mutant containing the reverted mutAB allele (ΔmutAB::mutAB ΔprpDC).
Effect of vitamin B$_{12}$ (cyanocobalamin) concentration on growth of the ΔprpDC mutant on propionate.
The concentration of vitamin B$_{12}$ was varied between 1 - 20µg/ml and the corresponding growth rate was plotted as a function of OD$_{600}$/ time.

4.7 Determining propionyl-CoA partitioning through the methylcitrate cycle and methylmalonyl-CoA pathway by gene expression analysis

The results from sections 4.3, 4.5 and 4.6 confirmed that both the methylcitrate cycle and the methylmalonyl pathway are functional in MTB. The individual operation of either pathway allows for growth on propionate (Fig. 5b and Fig. 12). However the relative contribution of either pathway to growth on vitamin B$_{12}$-supplemented propionate was not known. In order to ascertain if the enhanced growth on vitamin B$_{12}$ supplemented propionate relative to propionate (Fig. 5b ii and iii) was ascribed to the activity of both pathways, quantitative gene expression analysis by qRT-PCR was performed on selected genes from the two pathways (Fig. 15a). Levels of prpD, icl and mutB transcript were determined for bacteria cultured in propionate by real-time qRT-PCR and normalised against the values obtained from bacteria grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol, OADC enrichment and 0.05% Tween 80 to assess any differential regulation of these genes in propionate vs 7H9. Further the effects of vitamin B$_{12}$ on expression levels of these genes in propionate was evaluated. The methylcitrate cycle (prpD, icl) was transcriptionally responsive to a functional
methyImalonyl pathway as significantly different transcript profiles in the presence and absence of vitamin B$_{12}$ were obtained (Fig. 15). A significant reduction in prpD ($P < 0.0001$) and icl ($P < 0.0005$) expression levels was observed concomitant with vitamin B$_{12}$ supplementation of the propionate-containing medium (Fig. 15b). Using transcript levels as a proxy for flux, the inference that significantly lower transcript levels of prpD in propionate with vitamin B$_{12}$ (Fig. 15) corresponds to reduced flux through the methylcitrate cycle may be made.

Paradoxically, mutB transcript is more abundant in propionate media relative to propionate supplemented with vitamin B$_{12}$ (Fig. 15). The standard deviation for fold induction for mutB (Fig. 15) may reflect a high degree of heterogeneity in the sample population. It is also possible that raised expression of mutB in propionate in the absence of vitamin B$_{12}$ is a compensatory response illustrating the bacterium’s efforts for achieving optimal growth on this fatty acid.
Fig. 15 Quantitative expression analysis of methylcitrate cycle and methylmalonyl pathway genes in MTB

a. Expression of *prpD, icl* and *mutB* genes of H37Rv cultured in propionate compared to 7H9.

H37Rv with (■) and without (□) vitamin B$_{12}$. Insets denote expression of *prpD, icl* and *mutB* genes of H37Rv grown on propionate in the presence or absence of vitamin B$_{12}$ compared to 7H9. Significant differences in expression of *prpD* and *icl* in fatty acid carbon sources relative to the 7H9 control are denoted by a double asterisk ($P < 0.0005$) or single asterisk ($P < 0.005$).

b. Using the data represented in insets from a. the fold induction of *prpD, icl* and *mutB* genes of H37Rv cultured in propionate compared to propionate supplemented with vitamin B$_{12}$ is quantified.

Comparative analysis in expression of *prpD, icl* and *mutB* transcript of H37Rv grown in propionate media in the presence and absence of vitamin B$_{12}$. Significant differences in expression of *prpD* and *icl* in propionate relative to propionate with vitamin B$_{12}$ are denoted by a double asterisk ($P < 0.0001$) or single asterisk ($P < 0.0005$).

4.8 Growth of MTB on propionate in the absence of the glyoxylate cycle

Some microbes that metabolise propionate via the methylcitrate cycle require the glyoxylate cycle for carbon anaplerosis (Textor *et al.*, 1997; Wang *et al.*, 2003; Wegener *et al.*, 1969). By definition, anaplerotic reactions are those that form intermediates of the TCA cycle. For example, pyruvate, the end product of the methylcitrate cycle, could be channeled into the TCA cycle metabolite pool by other compensatory anaplerotic enzymes which use pyruvate as a substrate, such as pyruvate carboxylase (*pca*) and malic enzyme (*mez*) (Fig. 1). Alternately conversion to acetyl-CoA by pyruvate dehydrogenase (PDH) would depend on anaplerosis by the glyoxylate route, contingent on ICL (Fig. 1). TCA cycle intermediates could also be replenished by the activities of glutamate dehydrogenase and methylmalonyl-CoA mutase. In MSM, the anaplerotic enzyme, PCA, is part of the same operon as the methylcitrate cycle genes, which may allow for growth on propionate in the absence of the glyoxylate cycle, i.e. a $\Delta icl1\Delta icl2$ mutant of MSM can grow on propionate (Upton and McKinney, 2007). In *S. typhimurium*, which similarly does not require the glyoxylate cycle for growth on propionate, pyruvate is converted to oxaloacetate by the sequential activity of PEP synthase and PEP carboxylase (Horswill and Escalante-Semerena, 1999). As MTB possesses a bifunctional ICL,
it is unknown whether this enzyme acts only in its capacity as a MCL for growth on propionate in conjunction with anaplerotic enzymes, *pea* and *mez* (Fig. 1).

The advantages of having a propionate oxidizing pathway operate autonomously and independently from these other recognised anaplerotic enzyme was considered. Presumably, given that propionyl-CoA is converted to the TCA cycle intermediate, succinyl-CoA, by the methylmalonyl pathway, the activities of the other intact anaplerotic enzymes would be negligible.

The question of whether the methylmalonyl pathway was able to support growth in the absence of the bifunctional ICL/MCL enzyme was therefore investigated. This line of investigation was prompted by significant downregulation of not only *prpD* but also *icl* transcript in the presence of vitamin B<sub>12</sub>. Having established that vitamin B<sub>12</sub> supplementation could complement growth of the Δ*prpDC* mutant of H37Rv, the same remained to be demonstrated for Δ*icl1*. The lack of a complete methylcitrate and glyoxylate cycle in an ICL1 mutant was proposed not to have any consequence in the presence of vitamin B<sub>12</sub>, given the above mentioned rationale. Surprisingly, however, MCM-mediated growth of the Δ*icl1* mutant on propionate with vitamin B<sub>12</sub> supplement did not phenocopy that of the Δ*prpDC* mutant (Fig. 16). A two week lag phase was observed prior to the initiation of logarithmic growth. In other organisms, growth inhibition on propionate in MCL mutants was shown to be due to the accumulation of 2-methylcitrate and/or 2-methylisocitrate, earlier intermediates in the methylcitrate cycle, which competitively inhibit the NADP-dependent isocitrate dehydrogenase (Brock, 2005; Cheema-Dhadli et al., 1975; Horswill et al., 2001; Plaut et al., 1975). Similarly, it was speculated that in the absence of MCL activity, the buildup of intermediates from the methylcitrate cycle might account for the differences in growth of the Δ*icl1* and Δ*prpDC* mutants on vitamin B<sub>12</sub>-supplemented propionate. To test this idea, growth of ~Δ*prpDCΔicl1* (Δ*prpDC* grown in the presence of 3NP as described below), Δ*icl1* and Δ*prpDC* were compared on vitamin B<sub>12</sub>-supplemented propionate.
Fig. 16 The methylmalonyl pathway enables bypass of the glyoxylate cycle allowing growth of Δicl1 on propionate supplemented with vitamin B_{12}.
Growth of Δicl1 on propionate supplemented with vitamin B_{12} was compared to ΔprpDC and H37Rv. ▲, Δicl1; △, ΔprpDC; ■, H37Rv; ◆, Δicl1::icl1

3NP is a structural analog of succinate and a potent inhibitor of ICL (Honer Zu Bentrup et al., 1999; Schloss and Cleland, 1982). As such, 3NP was found to be inhibitory to growth of MTB Erdman on acetate and propionate but not on glucose (Muñoz-Elias and McKinney, 2005; Sharma et al., 2000). Consistent with the findings in MTB Erdman, (Muñoz-Elias and McKinney, 2005) H37Rv was similarly unable to metabolise propionate in the presence of 3NP (Fig. 17a). The growth of H37Rv with 3NP and vitamin B_{12} (Fig. 17a) correlated with growth of Δicl1 (Fig. 16), displaying a consistent two-week growth delay in propionate with vitamin B_{12}. This observation supported the specific inhibition of ICL by 3NP. Significantly, growth of ΔprpDC with vitamin B_{12} in the presence of 3NP was indistinguishable from growth of ΔprpDC with vitamin B_{12} supplement in the absence of 3NP (Fig. 17a). Since inactivation of *prpDC* precluded flux of propionyl-CoA through the methylcitrate cycle, the differential response of wild-type and ΔprpDC to 3NP-mediated inhibition of ICL (MCL),
implicated the accumulation of toxic, growth inhibitory methylcitrate cycle intermediates in 3NP-treated wildtype but not ΔprpDC strains, accounting for the lag observed in the former.

Similar growth rates during log phase growth on propionate with vitamin B$_{12}$ and 3NP were displayed by ΔprpDC and H37Rv (synonymous with the Δicl1 mutant) (Fig. 16 and Fig. 17a). This confirms the theory that the anaplerotic methylmalonyl pathway alone is sufficient for growth of MTB on propionate with vitamin B$_{12}$. Abrogation of MCM activity in the ΔmutAB mutant eliminated growth on propionate with vitamin B$_{12}$ in the presence of 3NP (Fig. 17b) confirming that the effects of vitamin B$_{12}$ supplementation were specifically mediated through MCM activity.

**Fig. 17** The methylmalonyl pathway enables bypass of the glyoxylate cycle during growth of MTB on propionate.

a. Growth of H37Rv on propionate in the presence of 3NP with (■) or without (□) vitamin B$_{12}$ supplementation vs. ΔprpDC on vitamin B$_{12}$-supplemented propionate with (▲) or without (△) 3NP.

b. Growth of the ΔmutAB mutant on vitamin B$_{12}$-supplemented propionate with (♦) or without (◆) 3NP
4.9 Emergence of suppressor mutants in response to metabolite stress

To examine whether the growth that was observed following the two-week lag was due to that of a suppressor mutant, a ‘reconstruction’ experiment was performed (Rosenberg, 2001). Cultures of H37Rv grown on propionate with vitamin B$_{12}$ and 3NP (Fig. 17a) and those of $\Delta icl1$ grown on propionate with vitamin B$_{12}$ (Fig. 16) were passaged several times in 7H9 media to eliminate residual traces of vitamin B$_{12}$. These cultures were then used as inoculum into media where the conditions of the original experiment where reconstructed i.e. an aliquot of the passaged culture was used to inoculate vitamin B$_{12}$-supplemented propionate with 0.1mM 3NP. In the case of $\Delta icl$, no 3NP was added. In both instances, growth rates after subculturing were comparable to that of H37Rv in vitamin B$_{12}$-supplemented propionate without 3NP (Fig. 18 and data not shown), indicating that the growth observed in the original experiment was due to the presence of a suppressor mutant. Importantly, the two-week growth delay was eliminated and growth remained strictly vitamin B$_{12}$ dependent. These observations suggested that a functional methylmalonyl pathway had allowed suppressor mutants to arise from 3NP inhibited cultures of H37Rv (and from $\Delta icl1$; data not shown) possibly as a result of toxic metabolite accumulation imposing stress-induced mutagenesis.
Fig. 18 Vitamin B$_{12}$-dependent growth in propionate of a suppressor mutant after serial passage in 7H9

H37Rv suppressor mutants isolated from vitamin B$_{12}$ supplemented propionate with 3NP were serially passaged in 7H9 media and reintroduced into vitamin B$_{12}$ supplemented propionate with ( nâ ) and without ( â ) 3NP. No growth of H37Rv suppressor mutants was observed in propionate with 3NP only ( â„ ).

Since the two-week delay was reproducible and observed independently with both the H37Rv and âicl1 strains, this suggested that mutants were not preexisting in the population as this would have been expected to result in variable growth kinetics. Assuming $\sim 6 \times 10^5$ CFU/ml (as determined from cultures seeded at OD=0.01, results not shown) and a spontaneous mutation frequency of $10^{-6} - 10^{-8}$, the emergence of mutants after 2 weeks would be feasible.

To determine the molecular basis of this heritable genotypic adaptation, the prpDC locus of suppressor mutants which arose from cultures of H37Rv grown in propionate with 3NP and vitamin B$_{12}$ (Fig. 17a) as well as âicl1 suppressor mutants grown in propionate with vitamin B$_{12}$ (Fig. 16), was sequenced to identify any mutations, as mutations in the prpDC operon and/or its promoter region might be expected to recapitulate the âprpDC growth phenotype on vitamin B$_{12}$ supplemented propionate with or without 3NP (Fig. 17a). However, no mutations were found in the prpDC genes or in the intergenic region between Rv1129c and Rv1130 (sequenced â500bp upstream of the Rv1130 ATG start codon spanning one third of Rv1129c). As a result, the mechanism for bypass of ICL1 essentiality in the wild type strain during growth on propionate and vitamin B$_{12}$ has yet to be identified.

Damage-induced mutagenesis is mediated in MTB by the actions of specialised DNA polymerases, such as DnaE2 (Boshoff et al., 2003). In addition, DinB1 and DinB2 are MTB homologues of E. coli polIV (DinB) which has been implicated in ‘adaptive’ or stress-induced mutagenesis in that organism (Goodman, 2002). Therefore, to investigate the role, if any, of specialised DNA polymerases in the emergence of the suppressor mutants described above, the ability of strains deficient in these DNA polymerases to overcome the growth impairment on propionate supplemented with vitamin B$_{12}$ and 3NP was tested. However, no differences in growth on propionate with vitamin B$_{12}$ and 3NP were observed between wild type, dnaE2-deficient and dinB1/dinB2-deficient strains were observed. A consistent, two-week growth
delay was observed in all strains (results not shown) further supporting the notion of a lack of pre-existing mutants in the starting culture and excluding a role for any of the specialised polymerases in induced mutagenesis by toxic methylcitrate cycle metabolite accumulation.

4.10 Growth on C₅ and C₁₇ carboxylic acids

Oxidation of longer odd-chain fatty acids releases derivative acetyl-CoA as well as propionyl-CoA subunits. In this scenario, the potential of an active methylmalonyl pathway was considered. Should propionyl-CoA be diverted into the methylmalonyl pathway, ICL would be relieved of its role as a MCL, improving efficiency of the glyoxylate cycle. This ‘division of labor’ was proposed to enable the optimal use of such carbon sources. Therefore growth on longer odd-chain fatty acids was assessed.

4.10.1 Growth on valerate

Growth on valerate (C₅) yields a 1:1 ratio of acetyl-CoA:propionyl-CoA. H37Rv was found to grow poorly on valerate in the absence of vitamin B₁₂ (Fig. 19a).
Fig. 19 Growth of MTB on valerate as a propionyl-CoA precursor

a. Stimulatory effect of vitamin B$_{12}$ on the growth of H37Rv and the ΔprpDC mutant on valerate (C$_{5}$). H37Rv with (◊) or without (●) vitamin B$_{12}$; ΔprpDC with (□) or without (■) vitamin B$_{12}$.

b. Improved growth of H37Rv on vitamin B$_{12}$-supplemented valerate is mutAB-dependent. ΔmutAB with vitamin B$_{12}$ (Δ); ΔmutAB containing the reverted mutAB allele with vitamin B$_{12}$ (ΔmutAB::mutAB) (●); H37Rv with (□) or without (■) vitamin B$_{12}$

In the absence of propionyl-CoA metabolism through the methylmalonyl pathway, catabolism of both propionyl-CoA and acetyl-CoA through the methylcitrate and glyoxylate routes respectively, may result in a ‘bottleneck effect’, where a degree of toxic intermediate buildup as a result of inefficient streaming of these metabolites may possibly account for the retarded growth. This observation together with the significant downregulation of methylcitrate cycle genes in the presence of a functional methylmalonyl pathway on propionate (Fig. 15) strongly implied that partitioning of propionyl-CoA through the methylmalonyl pathway might avoid cytotoxic effects by methylcitrate cycle intermediates on valerate supplemented with vitamin B$_{12}$. Indeed, growth was markedly improved on valerate supplemented with vitamin B$_{12}$ supporting this theory (Fig. 19a).

The prpDC mutant of H37Rv could not grow on valerate, as established previously by Muñoz-Elías et al. for the Erdman strain (Muñoz-Elías et al., 2006). However, supplementation with vitamin B$_{12}$ restored growth of ΔprpDC (Fig. 19a) presumably alleviating propionate toxicity resulting from the absence of both the methylcitrate cycle and methylmalonyl pathway. It was shown that the vitamin B$_{12}$-dependent growth on valerate is mediated by mutAB and furthermore, that ΔmutAB with vitamin B$_{12}$ on valerate grows as poorly as H37Rv without vitamin B$_{12}$, which again confirms that vitamin B$_{12}$ itself is not a regulator of the dynamic between these two propionate oxidizing pathways (Fig. 19b).
4.10.2 Comparative analyses of prpD, icl and mutB expression on different carbon sources

Inspection of prpD, icl and mutB transcript levels during logarithmic growth on valerate with vitamin B$_{12}$ indicated that levels of prpD and icl were further repressed than on propionate with vitamin B$_{12}$ (Fig. 20)

![Fig. 20 Expression of prpD, icl and mutB genes of H37Rv cultured on propionate or valerate in the presence of vitamin B$_{12}$](image)

Levels of prpD, icl and mutB transcript were determined by real-time qRT-PCR and normalised against the values obtained from bacteria grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol, OADC enrichment and 0.05% Tween 80 (7H9) to assess any differential regulation of these genes as a function of carbon source. Significant differences in expression of prpD and icl in fatty acid carbon sources relative to the 7H9 control are denoted by a single asterisk ($P < 0.0001$) or a double asterisk ($P < 0.005$).

This indicated that not only was propionyl-CoA catabolism being regulated largely by methylmalonyl pathway, as interpreted by low prpD levels, but also that the glyoxylate cycle
may not be active given the low \( icl \) levels. To test this, the growth of cultures on valerate in the presence of 3NP with and without vitamin B\(_{12} \) was assessed (Fig. 21a). This experiment showed that the effects of addition of 3NP to cultures grown on valerate were negligible in the presence of vitamin B\(_{12} \) supplement and again confirmed that vitamin B\(_{12} \)-dependent growth on valerate in the presence of 3NP is mediated by \( mutAB \) (Fig. 21b).

Whereas H37Rv was unable to grow (even partially) on valerate in the presence of the ICL inhibitor 3NP, growth of H37Rv with 3NP and vitamin B\(_{12} \) supplement was identical to that of \( \Delta prpDC \) with 3NP and vitamin B\(_{12} \) (Fig. 21a). The absence of differential growth between (H37Rv + vitamin B\(_{12} \) + 3NP) and (\( \Delta prpDC \) + vitamin B\(_{12} \) + 3NP) was in stark contrast to the differential response on propionate (Fig. 17a).

Fig. 21 Anaplerotic role for MCM revealed by growth of MTB on valerate with 3NP and vitamin B\(_{12} \) supplementation.

a. Growth of H37Rv on valerate in the presence of 3NP with (□) or without ( ■ ) vitamin B\(_{12} \) supplementation vs. \( \Delta prpDC \) on vitamin B\(_{12} \)-supplemented valerate with (Δ) or without ( ▲ ) 3NP.

b. Growth of \( \Delta mutAB \) (Δ) and \( \Delta mutAB::mutAB \) (▲) on vitamin B\(_{12} \)-supplemented valerate with 3NP.
This firstly implied that there was virtually no flux through the methylcitrate cycle which would presumably result in toxic intermediate buildup in the absence of ICL activity. The significant difference ($P < 0.0001$) in prpD levels between cultures grown on propionate with vitamin B$_{12}$ relative to valerate with vitamin B$_{12}$ (Fig. 20) coupled with the difference in growth phenotype of H37Rv with 3NP on vitamin B$_{12}$ supplemented propionate vs. valerate suggested that transcript levels were reflective of flux. Notably, the expression level of prpD in valerate supplemented with vitamin B$_{12}$ was not significantly different from that observed in 7H9 broth (Fig. 20). Secondly, it implied the capacity of the methylmalonyl pathway to perform an anaplerotic function for assimilation of acetyl-CoA in the absence of the glyoxylate cycle. The apparent dispensability of ICL for growth of MTB on valerate was confirmed by identical growths of ΔprpDC in the presence or absence of 3NP (Fig. 21a). This was reinforced by the observation that the addition of 3NP had no effect on growth of H37Rv in the presence of vitamin B$_{12}$ supplement (Fig. 22).

**Fig. 22** The operation of an autonomous methylmalonyl pathway bypasses the requirement for the glyoxylate cycle during growth of MTB on valerate

Growth of H37Rv on vitamin B$_{12}$-supplemented valerate with (□) or without (■) 3NP.
Paradoxically, the Δicll mutant was found to be unable to grow on valerate in the presence of vitamin B₁₂ (results not shown). The ICL enzyme may have some regulatory role on longer chain fatty acids which is important for isocitrate homeostasis at the ICL/ICD branchpoint, which had been disrupted in Δicll.

4.10.3 Growth on heptadecanoate

Growth of H37Rv and the prpDC mutant was then assessed on the longer chain fatty acid, heptadecanoate (C₁₇), the oxidation of which produces seven molecules of acetyl-CoA for each molecule of propionyl-CoA (7:1 ratio). In contrast to the findings on valerate, growth of H37Rv in the absence or presence of vitamin B₁₂ was indistinguishable (Fig. 23). Furthermore, unlike growth on propionate (Fig. 11) and valerate (Fig. 19a), the ΔprpDC grew on heptadecanoate in the absence of vitamin B₁₂ (Fig. 23), although initially, at a slightly slower rate than wild type. Vitamin B₁₂ supplementation augmented growth of ΔprpDC on heptadecanoate. This implied that in the presence of a functional methylmalonyl pathway, propionyl-CoA may be predominantly catabolised through this route, as seemed to be the trend in cultures grown in propionate and valerate with vitamin B₁₂ supplement. This would suggest that even though a functional methylmalonyl pathway appears dispensable for optimal growth on this carbon source as no visible growth advantage was afforded by vitamin B₁₂ for H37Rv as seen on propionate (Fig. 5b. ii and iii) and valerate (Fig. 19a), the flux distribution through these central networks may differ in vitamin B₁₂-supplemented vs. non-supplemented cultures. The anaplerotic contribution by a functional methylmalonyl pathway was not assessed, although it is probable that with increasing molar ratio of acetyl-CoA to propionyl-CoA, anaplerosis by the methylmalonyl pathway progressively declines consistent with the dispensability of this pathway for growth of ΔprpDC on longer odd-chain fatty acids.
Fig. 23 Growth of MTB on heptadecanoate (C\textsubscript{17})
Growth of H37Rv with (◊) or without (●) vitamin B\textsubscript{12}; growth of ΔprpDC on heptadecanoate (C\textsubscript{17}) both with (□) and without (■) vitamin B\textsubscript{12}.

It was considered that intracellular propionyl-CoA generated by the catabolism of heptadecanoate may be incorporated in methyl-branched lipids in ΔprpDC grown without vitamin B\textsubscript{12}. By extension, H37Rv grown on valerate may similarly divert propionyl-CoA into methyl-branched fatty acids. The activated methylmalonyl-CoA derivative from propionyl-CoA was shown to be used as a substrate by M. tuberculosis var. bovis cell extract to generate methyl-branched mycocerosic acids, providing direct evidence for elongation of preformed n- fatty acid primers with methylmalonyl-CoA (Rainwater and Kolattukudy, 1983). Each molecule of PDIM synthesised abstracts ten molecules of methylmalonyl-CoA from the intracellular metabolite pool (Trivedi et al., 2005). Growth on valerate was thus compared between isolates of H37Rv producing either negligible amounts of PDIM (the standard laboratory strain employed in the MMRU (Kana et al., 2008)) or significant amounts of this lipid (H37Rv strain received as a kind gift from Prof. C. Sassetti. University of Massachusetts). The rationale for this comparison was that if the latter strain could incorporate
greater amounts of propionyl-CoA levels into this methyl-branched virulence lipid than the former, this would translate into improved growth on valerate without vitamin B$_{12}$. The results demonstrated that the high PDIM-producing isolate of H37Rv showed markedly improved growth on valerate that is independent of the requirement for vitamin B$_{12}$ on this carbon source, compared to the poor PDIM producer strain employed in this study (Fig. 24). Growth of the two H37Rv strains on glycerol as a carbon source is identical. These results suggests that PDIM production may act to detoxify intracellular propionyl-CoA in MTB.

Fig. 24 Growth comparison on valerate between two variable PDIM-producing strains of H37Rv
Differential growth of H37Rv producing lower (■) and higher (□) levels of PDIM.

4.11 Growth of MTB on acetate with vitamin B$_{12}$

Growth of MTB on acetate requires the glyoxylate shunt (McKinney et al., 2000; Muñoz-Elias and McKinney, 2005). Alternate acetate assimilation pathways have been described, and a common denominator is that all such characterised pathways require MCM activity (Alber et al., 2006; Erb et al., 2007; Erb et al., 2008; Ivanovsky et al., 1997; Korotkova et al., 2002; Korotkova et al., 2005). Assessing the MTB genome for alternative acetate assimilation
pathways using bioinformatics is complicated as the enzyme activities for all of the steps in these pathways have yet to be resolved. The possibility that MTB could utilise acetate in the absence of the glyoxylate shunt was therefore investigated experimentally. Acetate supplemented with vitamin B$_{12}$ (to enable MCM) and 3NP (to inhibit ICL) did not support growth of MTB (Table 2). The results in Table 2 suggest that alternate acetate assimilation pathways are either not expressed or are not functional in MTB under standard growth conditions.

<table>
<thead>
<tr>
<th></th>
<th>Acetate</th>
<th>Acetate + 3NP</th>
<th>Acetate + vitamin B$_{12}$ + 3NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Growth on MTB on acetate supplemented with 3NP and vitamin B$_{12}$.
MTB grew on acetate media (+), but failed to grow in the presence of 3NP (-) regardless of vitamin B$_{12}$ supplementation.
5 DISCUSSION

In this work, mutants of MTB H37Rv impairing one or both of the recognised pathways for propionate oxidation were generated by allelic exchange mutagenesis. Flux through either pathway enabled growth of the mycobacterium on propionate, suggesting a redundancy of function for growth on propionate. Inhibition of both pathways abrogated growth on propionate, suggesting the presence of only these two functional pathway alternatives for propionyl-CoA catabolism in vitro (Fig. 2).

5.1 The MTB gene complement for propionate oxidation

In silico interrogation of the whole genome sequence of MTB indicated that this organism possesses two possible routes for the catabolism of propionyl-CoA, namely the methylcitrate cycle and the methylmalonyl pathway. All putative components of the methylmalonyl pathway were readily identified as being intact by homology searches suggesting a complete and functional pathway. Conversely, the methylcitrate cycle appeared incomplete as it lacked a homologue of prpB, which encodes MCL. MTB does not have the characteristic prpDBC gene cluster present in other actinomycetes such as C. glutamicum (Claes et al., 2002) and MSM (Upton and McKinney, 2007) and appears to have undergone a probable deletion of prpB in this region. M. leprae, on the other hand, has lost the entire prpDBC operon but retains a complete MCM pathway (Cole et al., 2001). Potentially contributing to the apparent loss of prpB in MTB would be the presence of another protein having MCL function. A bifunctional ICL possessing MCL/ICL activity was considered as one possibility, as reported for several organisms (Brock, 2005; Horswill and Escalante-Semerena, 1999; Luttik et al., 2000; McFadden et al., 1972; Pronk et al., 1994; Upton and McKinney, 2007). Another possibility was that PrpB activity in MTB is provided by Rv1998c which was identified as a member of the group of proteins included in COG2513. This group of proteins is part of the ICL/PEPM enzyme superfamily. Significantly, a member of COG2513 is E. coli PrpB, which
was the template with highest homology to Rv1998c selected by the Robetta server for modeling the tertiary structure of this CHP. On the basis of these findings, Rv1998c was hypothesized to provide non-orthologous MCL activity in MTB.

5.2 MCM is not essential for propionate metabolism in MTB

To assess the role of the methylmalonyl pathway in MTB H37Rv, the MCM-encoding *mutAB* genes were functionally inactivated and the mutant was assessed for growth on propionate. Surprisingly, no difference in propionate metabolism was observed between the parental and Δ*mutAB* strains, indicating that the methylmalonyl pathway is dispensable for growth on propionate (Fig. 5) and confirming the existence of an alternate pathway(s) for propionate metabolism, specifically, the methylcitrate cycle (see section 5.5). This finding contrasts with the situation in the actinomycetes *Nocardia corallina*, *Aeromicrobium erythreum* and *S. cinnamonensis*, where disruption of *mutB* abolished growth on propionate (Reeves *et al.*, 2004; Valentin and Dennis, 1996; Vrijbloed *et al.*, 1999), indicating that, unlike MTB, these actinomycetes do not possess alternative pathways for propionate metabolism or that these pathways are negatively regulated by vitamin B_{12}.

5.3 Vitamin B_{12} supplementation facilitates operation of the methylmalonyl pathway

Despite reports that MTB has a complete gene complement for coenzyme B_{12} biosynthesis (Rodionov *et al.*, 2003), the results of a related study suggested that MTB does not synthesise the cobalamin cofactor *in vitro* (outside the living host). In that study, vitamin B_{12} supplementation was shown to be required to support the function of the vitamin B_{12}-dependent methionine synthase, MetH (Warner *et al.*, 2007). As vitamin B_{12} is also required as a cofactor for MCM, cyanocobalamin was included as a supplement in the growth medium and was found to stimulate growth of MTB on propionate (Fig. 5b. ii and iii). It was evident that increasing concentrations of propionate were metabolised less efficiently by the methylcitrate cycle in the absence of vitamin B_{12} supplement. Two possible explanations are proposed. (i) Higher concentrations of propionate require the synthesis of TCA cycle intermediates via induction of glyoxylate cycle enzymes in addition to the reliance on
anaplerotic enzymes such as malic enzyme or PCA (Muñoz-Elias et al., 2006) (ii) Should oxidation of propionyl-CoA via the methylcitrate cycle proceed to acetyl-CoA via the pyruvate dehydrogenase complex, inhibition of this enzyme complex by elevated levels of propionyl-CoA (Brock and Buckel, 2004; Maerker et al., 2005; Maruyama and Kitamura, 1985) may explain the poor initial growth on 0.2% propionate, as activation and turnover of propionate needs to be balanced (Brock and Buckel, 2004). Supplementation with vitamin B$_{12}$ eliminates this lag, consistent with the elimination, by supplementation with succinate, of an extended lag phase similarly displayed in *E. coli* cultures grown on propionate (Wegener et al., 1968a).

The impaired growth of ΔmutAB on 0.1 and 0.2% propionate supplemented with vitamin B$_{12}$ relative to H37Rv indicated that the improved growth was mediated by MCM (Fig. 5b. ii and iii) and provided the first evidence that all of the reactions required for a complete methylmalonyl pathway are functional in MTB. Further, the identical growth of ΔmutAB on propionate in the presence of vitamin B$_{12}$ to H37Rv grown on propionate in the absence of vitamin B$_{12}$ (Fig. 5b. ii) indicates that the alternate pathway(s) for propionate assimilation are not negatively regulated by vitamin B$_{12}$. However, although the independent operation of either propionate oxidation pathway in MTB is considered unlikely, from these observations alone it is not possible to determine whether the enhanced growth of H37Rv on vitamin B$_{12}$-supplemented propionate reflects operation of solely the MCM pathway, or a combination of pathways. Thus, further investigation of the methylmalonyl pathway in MTB was dependent on a concomitant investigation of the operation of the methylcitrate cycle. This study therefore probed the possible contribution of Rv1998c activity to propionate metabolism in MTB in parallel with studies on the methylmalonyl pathway.

### 5.4 The unresolved function of Rv1998c in MTB

Growth of the ΔmutAB mutant on propionate (Fig. 5) provided the first evidence that the putative methylcitrate cycle identified using bioinformatic analysis (section 4.4) was responsible for growth on this substrate in the absence of a functional methylmalonyl pathway.
Early reports that ICL1 and, to a lesser extent, ICL2 are required for growth on propionate in MTB (Muñoz-Elias and McKinney, 2005) could be interpreted in two ways. The first implicates a role for these enzymes as MCLs even though it had been reported that ICL cannot substitute for MCL for growth on propionate in other organisms (Bramer and Steinbuchel, 2001; Brock, 2005; Claes et al., 2002; Horswill and Escalante-Semerena, 1999). The second is that the operation of the methylcitrate and glyoxylate cycles together are required for growth on propionate (Textor et al., 1997; Wang et al., 2003; Wegener et al., 1969) and that MCL activity is not provided by ICL. Rv1998c was identified as a potential candidate for providing MCL activity in MTB (Fig. 6 and Fig. 7). Studies reported during the course of this work subsequently revealed that ICL1 from MTB Erdman does indeed have dual ICL and MCL activity (Gould et al., 2006; Muñoz-Elias et al., 2006) although recombinant ICL2 had no MCL activity. Therefore, the poor growth of an Δicl1 mutant on propionate could be as a result of another uncharacterised enzyme functioning as a MCL in the methylcitrate cycle with the further requirement for ICL2 exclusively in the glyoxylate cycle, supporting the notion forwarded above (Muñoz-Elias and McKinney, 2005).

Rv1998c was initially over-expressed as a His-tag protein in E. coli and found to be insoluble under all conditions tested, in contrast to recombinant E. coli PrpB which was over-expressed in parallel as a soluble protein. The production of insoluble inclusion bodies when over-expressing recombinant protein in E. coli is particularly pronounced with MTB proteins (www.webtb.org). Further studies therefore focused on expression of Rv1998c in MTB under the control of a Tet-inducible promoter. Although RT-PCR analysis confirmed Tet-regulated over-expression of Rv1998c transcript in MTB, no MCL activity was detected in the crude cell extracts (Table 1). As expected, MCL activity was induced by growth of wild type MTB on propionate (Table 1; (Muñoz-Elias et al., 2006) thus validating the biochemical assay for MCL activity. The lack of activity could be due to inefficient translation of the Rv1998c transcript resulting in low protein levels. Alternatively, it is possible that Rv1998c protein was over-produced but has no MCL activity.

A genetic approach was thus adopted to further investigate whether Rv1998c possesses MCL activity. Growth on the reduced carbon source, palmitate, results in up-regulation of the DosR regulon in MTB (Boshoff et al., 2004). As a member of the DosR regulon (Voskuil et al.,
2003), induction of Rv1998c on propionate may account for the residual MCL activity observed in cell extracts from $\Delta icl1$ Erdman (Muñoz-Elias et al., 2006). It was postulated, therefore, that the poor growth of $\Delta icl1$ Erdman observed on propionate (Muñoz-Elias and McKinney, 2005) would be eliminated by loss of Rv1998c gene function. However, whereas H37Rv was readily transformed with the Rv1998c knockout construct to yield single crossover recombinants, no single crossovers were obtained in the $\Delta icl1$ mutant of Erdman consistent with the markedly reduced frequency of homologous recombination in the Erdman strain of MTB compared to the H37Rv strain employed in this study (G. Abrahams, unpublished; J. McKinney, personal communication).

An alternate hypothesis is that Rv1998c may play a role in propionate metabolism as part of a putative citramalate pathway in MTB. 2-Methylmalate is a metabolite in the citramalate pathway implicated in propionate oxidation (Textor et al., 1997), acetate assimilation (Ivanovsky et al., 1997) and autotrophic CO$_2$ fixation (Herter et al., 2002b) (Fig. 25).
Fig. 25: Citramalate cycle adapted from Ivanovsky et al (2002) and Textor et al (1997). The scheme shows the citramalate cycle for acetate assimilation (right to left in blue) where acetate and pyruvate are condensed to citramalate by Cms; citramalate synthase (Ivanovsky et al., 1997; Meister et al., 2005; Xu et al., 2004). Cms from Leptospira interrogans has highest homology (31% identity, 52% similarity) to hsaF (in the KstR regulon (Kendall et al., 2007b; Van der Geize et al., 2007) of MTB. Further the scheme shows the citramalate cycle for propionate oxidation (left to right in red) (Friedmann et al., 2007; Herter et al., 2002a; Textor et al., 1997) where propionyl CoA and glyoxylate are condensed to β-methylmalyl-CoA by Mcl; L-malyl-CoA lyase/β-methylmalyl-CoA (Meister et al., 2005). Mcl from R. capsulatus has highest homology (31% identity, 44% similarity) to the CHP Rv3075c of MTB. Following dehydration/hydration of β-methylmalyl-CoA by unknown enzymes to 2-methylmalate, cleavage to acetate and pyruvate is postulated by Rv1998c.

2-Methylmalate was identified as a substrate for PDP, a member of the ICL/PEPM enzyme superfamily whose broad substrate activity also includes 2-methylisocitrate, as mentioned previously. If Rv1998c displays similar diversity of function, it is possible MTB possesses a citramalate pathway that may function in propionate oxidation in MTB. To test this hypothesis, growth of the ΔprpDC mutant expressing Rv1998c under the control of a Tet-regulated promoter was assessed in propionate media. Tet-induced expression of Rv1998c was unable to facilitate growth of this mutant on propionate (results no shown) suggesting that further biochemical studies would be required to establish whether a citramalate pathway for propionate oxidation, having Rv1998c perform as a putative citramalate lyase, exists in MTB. Conceivably, genes of the citramalate pathway may be induced only under certain in vivo conditions.

### 5.5 Conditional essentiality of ΔprpDC for growth on propionate

During the course of this study, elegant work by others (Muñoz-Elias et al., 2006) demonstrated the essentiality of the methylcitrate cycle for growth of MTB Erdman on propionate. The contribution of the methylcitrate cycle to propionate metabolism in H37Rv MTB was therefore investigated by the construction of a mutant strain lacking the prpDC genes, which encode the first two enzymes of the cycle (Fig. 2). As observed in Erdman (Muñoz-Elias et al., 2006), loss of prpDC also abolished growth of H37Rv MTB on
propionate (Fig. 11). However, the conditional essentiality of the methylcitrate cycle *in vitro* (Fig. 11) was shown to be due to the vitamin B$_{12}$ auxotrophy of MTB, as growth of ΔprpDC on propionate was observed provided the media was supplemented with vitamin B$_{12}$ (Fig. 12). The fact that growth of ΔprpDC on propionate with vitamin B$_{12}$ supplement was abrogated by loss of mutAB function (Fig. 13) confirmed the essential role of the vitamin B$_{12}$-dependent activity of MCM in growth of the ΔprpDC mutant on propionate. Furthermore, the finding that supplementation with exogenous cyanocobalamin (vitamin B$_{12}$) is able to support the activity of MCM (Fig. 12 and Fig. 13) confirms that MTB can not only assimilate vitamin B$_{12}$ from the environment, but also has a functional ATR system for enzymatic conversion of vitamin B$_{12}$ to the active form of the cofactor. Together, these observations establish unambiguously the existence of a functional methylmalonyl pathway in MTB.

### 5.5.1 A functional methylmalonyl pathway in MTB

Propionate incorporation into resting cells of MTB (Yano and Kusunose, 1966) contributed to earlier evidence supporting the activity of PCC in mycobacteria. Subsequently, variable PCC activity was reported in MSM cultured under different conditions (Wheeler *et al*., 1992), for which the quaternary structure of the PCC complex was available (Haase *et al*., 1982). The main role of the recently characterised αAccA3-βAccD5-εAccE5 biotin-dependent PCC complex of MTB is proposed to be the synthesis of methylmalonyl-CoA (Gago *et al*., 2006; Oh *et al*., 2006). In MSM, the AccD5 orthologue, together with AccA3 and AccD4, comprise the subunits of the ACC involved in mycolic acid biosynthesis (Portevin *et al*., 2005), suggesting a broader role for the AccD5 carboxytransferase β-subunit in MTB. In *C. glutamicum*, linear chain ketoacyl derivatives are substrates for carboxylase complexes essential for mycolic acid synthesis (Gande *et al*., 2004; Gande *et al*., 2007); specifically, AccD2 and AccD3 constitute the β subunits of the carboxylase involved in mycolic acid synthesis (Gande *et al*., 2004; Gande *et al*., 2007). Supporting a broader role for AccD5 in mycolic acid biosynthesis, an accD2 mutant of *C. glutamicum*, the orthologue of accD5 in MTB (Gande *et al*., 2007) is deficient in mycolic acid production (Gande *et al*., 2004). Efforts
are been made to design inhibitors against the PCC of MTB (Lin et al., 2006b) using the crystal structure of AccD5 (Holton et al., 2006; Lin et al., 2006b), given the essentiality of the subunit components in vitro (Holton et al., 2006; Sassetti et al., 2003).

MCM requires the (R)-epimer of methylmalonyl-CoA as a substrate. The putative epimerase responsible for the conversion to this stereoisomer was identified as Rv1322A. However, the promiscuous $\alpha$-methylacyl CoA racemase (MCR ; Rv1143) which is intimately involved in the $\beta$-oxidation of methylbranched fatty acids originating from isoprenoids, has reported racemization activity with methylmalonyl-CoA thioester and might provide redundant activity. Its wide range of substrates includes pristanic acid. MCR is essential in mycobacteria for the oxidation of this carboxylic acid (Sakai et al., 2004). Conceivably, the propionyl-CoA liberated during $\beta$-oxidation is carboxylated to (S)-methylmalonyl-CoA, which is further dependent on this MCR for conversion to the corresponding (R)-methyl enantiomer in a reaction that is cobalt dependent, interestingly so, given the cobalt requirement for the $\text{B}_{12}$ coenzyme (Bhaumik et al., 2007). Should growth on vitamin $\text{B}_{12}$-supplemented propionate be abolished in a strain where both prpDC and Rv1322A have been deleted, redundant epimerase activity by MCR could be excluded.

Vitamin $\text{B}_{12}$ was titrated to establish the optimal concentration of this supplement for growth of the $\Delta$prpDC mutant on propionate (Fig. 14). However growth of $\Delta$prpDC equivalent to that of wildtype (with or without vitamin $\text{B}_{12}$) could not be obtained, even with saturating concentrations of vitamin $\text{B}_{12}$ (>7.5 $\mu$g/ml). The following possibilities were considered:

i. Propionate oxidation via the methylmalonyl pathway may not be as efficient as via the methylcitrate cycle as growth of $\Delta$prpDC with vitamin $\text{B}_{12}$ (Fig. 12) was poorer than wild type without vitamin $\text{B}_{12}$ (Fig. 11). However, this is unlikely in organisms where close co-operation of the methylcitrate and glyoxylate cycles exists (Textor et al., 1997; Wang et al., 2003; Wegener et al., 1969) as it undermines the rate of energy flow by circumventing two NADH-generating reactions of the TCA cycle, and also fails to harness the ATP-generating potential of the propionyl-CoA metabolite from substrate level phosphorylation at the succinyl-CoA synthase junction common to the
methylmalonyl pathway and TCA cycle (Hamblin et al., 2008). The methylmalonyl-CoA pathway therefore appears the more efficient route for propionate metabolism.

ii. The operation of the methylmalonyl pathway is constrained either by inefficient uptake or adenosylation of vitamin B\(_{12}\) which leads to insufficient coenzyme. However, a metE mutant grows as well as wild type suggesting efficient transport of vitamin B\(_{12}\) (Warner et al., 2007) despite the apparent absence of a specific high affinity transporter system (Rodionov et al., 2003). The B\(_{12}\)-dependent MetH and MCM enzymes require a different form of cofactor for activity, namely, methylcobalamin vs. adenosylcobalamin. Therefore, processing of exogenous vitamin B\(_{12}\) to the active coenzyme subsequent to uptake may rather be the rate-limiting step.

iii. Insufficiency in other aspects of the methylmalonyl pathway such as CO\(_2\) for carboxylation of propionate or constitutively low levels of MCM itself (see section 5.6) is a further possibility. Growth on propionate may be enhanced by the addition of biotin or sodium bicarbonate by stimulating PCC activity, as has been reported in mycobacteria (Wheeler et al., 1992) and other organisms (Evans et al., 1993; Maruyama and Kitamura, 1985). Preliminary examinations of \(\Delta\)prpDC grown in propionate with vitamin B\(_{12}\) in a 5% CO\(_2\) humidified incubator surprisingly revealed no growth for \(\Delta\)prpDC, although wild type cultured under identical conditions grew well (data not shown), consistent with the reported stimulatory effects of 5% CO\(_2\) on growth of MTB (Schaefer et al., 1955). A plausible explanation for these observations is that rate-limiting enzymes downstream of PCC may result in buildup of methylmalonyl-CoA as a result of the increased flux through this pathway. In Caenorhabditis elegans, accumulation of this metabolite produced increased amounts of growth inhibitory methylmalonic acid in the culture media (Chandler et al., 2006).

iv. Propionyl-CoA may, in the absence of prpC, competitively inhibit citrate synthase (Horswill et al., 2001; Man et al., 1995) counteracting the growth stimulatory effects of an active methylmalonyl pathway. Should this be the case, it is clear that the citrate synthase cannot be that proficient in making 2-methylcitrate as the low levels produced are not enough to arrest cell growth in the presence of vitamin B\(_{12}\).
The poorer growth on propionate supplemented with vitamin B\textsubscript{12} of both ∆prpDC (Fig. 12) and ∆mutAB mutants (Fig. 5b. ii) relative to the wild type strain (Fig. 5b. ii) reinforced the notion that the additive effects of two propionate oxidizing pathways are required for optimal growth on this fatty acid carbon source. If the methylmalonyl pathway alone was responsible for improved growth on propionate supplemented with vitamin B\textsubscript{12} (Fig. 5b. ii and iii) and if the methylcitrate pathway is negatively regulated by vitamin B\textsubscript{12}, then growth of the ∆prpDC mutant would be identical to that of the wild type with vitamin B\textsubscript{12} supplement and the ∆mutAB mutant would not be expected to grow. In the case of the two methionine synthases, MetE and MetH, their activity is mutually exclusive as exogenous vitamin B\textsubscript{12} represses transcription of metE by binding to the upstream B\textsubscript{12}-riboswitch motif present in the promoter region of this gene (Warner et al., 2007). Since only two B\textsubscript{12}-riboswitch motifs have been identified in the MTB genome, the other located immediately upstream of PPE2 (Rodionov et al., 2003; Vitreschak et al., 2003), the methylcitrate cycle in MTB is not subject to this regulatory mechanism (∆mutAB grows in the presence of vitamin B\textsubscript{12}, unlike ∆metH (Warner et al., 2007)). Thus the observations in this study suggested a synergistic interaction between the vitamin B\textsubscript{12}-dependent methylmalonyl pathway and vitamin B\textsubscript{12}-independent methylcitrate cycle. To further investigate this relationship, their regulation was therefore probed by gene expression analysis.

5.6 Transcriptional analysis of prpD, icl and mutB

Global regulation of gene expression by transcription factors (Martinez-Antonio and Collado-Vides, 2003) or bifunctional proteins active in both metabolism and control of gene expression, (known as trigger enzymes; (Commichau and Stulke, 2008)), is generally the mainstream control of flux distribution. In this study, the levels of transcript for selected genes in the methylmalonyl pathway and methylcitrate cycle were investigated as an indicator of intracellular flux distribution through the pathways. In other organisms, a correlation between gene expression and metabolic activity on propionate has generally been observed for the methylcitrate cycle (Claes et al., 2002; Huser et al., 2003; Plassmeier et al., 2007). In MTB,
the genes of the methylcitrate cycle (\textit{prpD} and \textit{icl}) were found to be significantly upregulated in propionate compared to propionate supplemented with vitamin B_{12} (Fig. 15). The reduced levels of \textit{prpD} and \textit{icl} transcript implied that the improved growth on B_{12}-supplemented propionate (Fig. 5b. ii and iii) may be largely due to the activity of the methylmalonyl pathway rather than upregulation of both pathways simultaneously. Paradoxically, however, growth of \textit{\Delta prpDC} (Fig. 12) is significantly poorer than wild type (Fig. 5b. ii and iii) on propionate media supplemented with vitamin B_{12}.

The downregulation of \textit{prpD} and \textit{icl} in response to vitamin B_{12} supplementation is interesting given the lack of an identifiable upstream B_{12}-riboswitch in the promoter regions of these genes. Instead, this transcriptional responsiveness is probably regulated by propionyl-CoA and derivative metabolite levels rather than by vitamin B_{12}, since \textit{\Delta mutAB} grows similarly on propionate in the presence and absence of this supplement (Fig. 5b. ii). If vitamin B_{12} was directly responsible for influencing a reduction in \textit{prpD} and \textit{icl} transcript, a reduced growth rate for \textit{\Delta mutAB} on B_{12}-supplemented propionate relative to propionate would be expected. Presumably, vitamin B_{12} facilitates propionate regulation indirectly by enabling MCM activity, which influences the intracellular metabolite profile.

However, it is recognised that induction at the transcriptional level does not always reflect the level of flux or enzyme activity in these pathways. This is exemplified by the fact that there was virtually no induction of \textit{mutB} in B_{12}-supplemented propionate cultures (Fig. 15) despite the modest contribution to growth by this pathway, as evidenced by growth of \textit{\Delta prpDC} in propionate with vitamin B_{12} (Fig. 12). This is contrary to the observed B_{12}-riboswitch located upstream of \textit{mutA} in \textit{P. shermanii} (Vitreschak \textit{et al.}, 2003), which suggests that in this actinomycete, MCM would be transcriptionally responsive to vitamin B_{12}. In MTB, the absence of induction may suggest that given the remarkably accelerated reaction rate of this enzyme (Chowdhury and Banerjee, 2000; Doll and Finke, 2003) constitutive levels of MCM suffice, which are able to support any measurable flux through the pathway. Furthermore, given the very low rate of generation of inactive mutases (Padovani and Banerjee, 2006; Vlasie and Banerjee, 2004; Yamanishi \textit{et al.}, 2005), a low, steady-state turnover of the protein is maintained. Consistent with the findings of this study, it is worth noting that numerous studies profiling the global transcription of MTB genes under different conditions have not
found mutAB to be highly responsive. Insertion of the mazEF-type TA module inserted between mutAB and the putative meaB of MTB may have selected for constitutive low expression of this operon, since fluctuations in mazEF transcript levels could conceivably result in an excess of the stable toxin over the unstable antitoxin with negative consequences for the bacterium. Tight regulation of mutAB might therefore account for the poorer growth of ΔprpDC relative to wildtype on vitamin B12-supplemented propionate.

In future studies, metabolic analysis using 1,2-[13C]-propionate could be done to distinguish the partitioning between these two pathways. Specifically, the coupling interaction of 13C-13C-12C of the propionate carbon skeleton would be preserved with flux through the methylcitrate cycle as it is converted to pyruvate, where it is lost in the randomizing methylmalonyl pathway, as demonstrated in E. coli (London et al., 1999). The relative contribution of these pathways to the formation of alanine (or its pyruvate precursor) could be calculated from the abundance of the different mass isotopes. Developments made in extraction protocols for metabolome analysis in mycobacteria make such future studies feasible (Jaki et al., 2006).

5.7 Bypassing the requirement for the glyoxylate cycle for growth on propionate by the methylmalonyl pathway

In this study, carbon anaplerosis by the methylmalonyl pathway independent of the glyoxylate cycle on the fatty acids, propionate and valerate (see section 5.9), was demonstrated. Growth on odd-chain fatty acids has been noted in the absence of virtually any glyoxylate cycle activity (Blevins and Perry, 1972; Evans et al., 1993) or in mutants defective for the glyoxylate cycle (Horswill and Escalante-Semerena, 1999; Nieder and Shapiro, 1975; Sakai et al., 2004; Upton and McKinney, 2007). These observations suggested that in some organisms, propionate metabolism is divorced from the glyoxylate cycle. In the absence of carbon anaplerosis through this pathway, other candidate anaplerotic enzymes were obviously supporting growth on this fatty acid although no studies have been done to elucidate which may have been responsible. By deduction, if propionate is metabolised to pyruvate through one of several previously described routes, malic enzyme, pyruvate carboxylase, or PEP
synthase and PEP carboxylase would be individually or collectively responsible for ‘topping up’ of TCA cycle intermediates (Fig. 1).

In this study, an icl1 mutant of MTB was shown to be able to grow on B_{12}-supplemented propionate in the absence of both the methycitrate and glyoxylate cycles (Fig. 16). Similarly, inhibition of ICL with 3NP allowed for growth of MTB on propionate subject to supplementation with vitamin B_{12} (Fig. 17a). This growth was shown to be mediated by mutAB (Fig. 17b). There are two known pathways which form C_{4} acids directly from propionate. For the first pathway, Reeves and Ajl (1962) reported the synthesis of α-hydroxyglutarate from propionyl-CoA and glyoxylate catalyzed by cell-free extracts from E. coli grown on propionate. α-Hydroxyglutarate is metabolised to succinate although no attempt was made to purify the enzyme and to elucidate its mechanism of action (Reeves and Ajl, 1962; Wegener et al., 1968b). α-Hydroxyglutarate synthase is not expected to function in the absence of ICL (Wegener et al., 1968a; Wegener et al., 1969). The second is the anaplerotic methylmalonyl pathway.

Despite the significantly reduced levels of prpD in propionate with vitamin B_{12} relative to propionate alone (Fig. 15), the methylcitrate cycle was still partially active as evidenced by the growth delays of Δicl1 in propionate with vitamin B_{12} and H37Rv in propionate with vitamin B_{12} and 3NP when compared to ΔprpDC, confirming that the growth delay was a result of the build-up of toxic 2-methylcitrate/2-methylisocitrate metabolites (Brock, 2005; Cheema-Dhadli et al., 1975; Horswill et al., 2001; Plaut et al., 1975). Similar toxic intermediate buildup has been reported by function impairment of the methylcitrate cycle in Ralstonia eutropha (Bramer and Steinbuchel, 2001), MSM (Upton and McKinney, 2007) and C. glutamicum (Plassmeier et al., 2007). However, this does not necessarily detract from the ability of the methylmalonyl pathway to support growth independently of ICL. Together, these observations provide conclusive evidence that the methylmalonyl pathway bypasses the need for anaplerosis via the glyoxylate cycle and is sufficient for the growth of MTB on propionate as the sole carbon source, provided that the vitamin B_{12} cofactor requirement for MCM activity are met.
Unlike MTB, MSM produces vitamin B\textsubscript{12} in vitro (Karasseva et al., 1977) (S. Dawes, personal communication). In MSM the growth of Δicl\textsubscript{1}Δicl\textsubscript{2}, ΔprpDBC and ΔprpDBCΔicl\textsubscript{1}Δicl\textsubscript{2} mutants on propionate (Upton and McKinney, 2007) could therefore be attributed to a putative methylmalonyl-CoA pathway (Stjernholm et al., 1962; Wheeler et al., 1992). A ΔprpDCΔmutAB mutant would confirm if the methylmalonyl pathway is functional and/or if another propionate oxidizing pathway is present in MSM. This is the focus of ongoing studies in the MMRU. Notably, 3NP inhibits growth of MSM on acetate (Muñoz-Elías, 2005). However, preliminary findings suggested that 3NP does not inhibit growth of a ΔmutAB mutant of MSM on propionate (data not shown) in contrast to the 3NP-mediated inhibition of MTB grown on this carbon source (Fig. 17). Assuming that propionate oxidation in MSM occurs only via the methylmalonyl pathway and methylcitrate cycle, these observation suggest that 3NP specifically inhibits ICL and not the MCL of MSM and that MSM is able to oxidise propionate in the absence of carbon anaplerosis by the glyoxylate cycle or methylmalonyl pathway. In this case, anaplerosis is presumably orchestrated by pca (Fig. 1) which, in MSM, is transcribed as part of the methylcitrate cycle gene operon (Mukhopadhyay and Purwantini, 2000). These findings contrast with the essential requirement of the glyoxylate cycle by some microorganisms during growth on propionate (Textor et al., 1997; Wang et al., 2003; Wegener et al., 1969).

### 5.8 Genotypic adaptation of Δicl\textsubscript{1} to growth on B\textsubscript{12}-supplemented propionate

As described above, toxic metabolite buildup could account for the retarded growth of Δicl\textsubscript{1} on propionate with vitamin B\textsubscript{12} (Fig. 16), H37Rv with 3NP on propionate with vitamin B\textsubscript{12} (Fig. 17a) and also, of H37Rv on valerate without vitamin B\textsubscript{12} (see section 5.9 below). Positive feedback by 2-methylcitrate is regulated by the transcriptional activator, PrpR, in S. enterica (Palacios and Escalante-Semerena, 2004). The genes for propionate oxidation in S. enterica are located in two adjacent divergently transcribed units, prpR and prpBCDE (Horswill and Escalante-Semerena, 1997). Closer examination of the prpDC locus in MTB displays a similar gene arrangement, having a 20bp intergenic region between prpDC and a putative transcriptional regulatory protein (Rv1129c) transcribed in the reverse orientation.
Rv1129c is induced (17-fold) with prpDC in activated macrophages (Schnappinger et al., 2003). A similar feedback mechanism may occur in MTB through buildup of 2-methylcitrate/2-methylisocitrate in the absence of ICL (Palacios and Escalante-Semerena, 2004). This may be particularly relevant to the attenuated phenotype observed for an ICL mutant in vivo (Muñoz-Elias and McKinney, 2005), and reinforces the importance of investigating the regulation of the methylcitrate cycle in MTB. As a result, ICL may be essential for its MCL activity rather than for its role in the glyoxylate or PEP-glyoxylate cycles. Supporting this possibility is the following: (i) unlike icl, glcB encoding MLS is not upregulated in the lungs of chronically infected mice (Timm et al., 2003); and (ii) the apparent attenuation of a ∆prpD but not a ∆prpDC in mice (Mattow et al., 2006). However, if this were the case, one would expect that an icl mutant would be as severely attenuated as ∆icl1∆icl2 (McKinney et al., 2000; Muñoz-Elias and McKinney, 2005) assuming that no MCL activity is afforded by ICL2. Should another enzyme function as a MCL (accounting for the growth of the ∆icl1 on propionate), then the suggestion that ICL is essential by virtue of its role as a MCL would be invalidated.

Genetic adaptation, presumably in response to metabolite stress, was revealed by isolating suppressor mutants from H37Rv grown in propionate with vitamin B₁₂ and 3NP (Fig. 17) as well as from ∆icl1 grown in propionate with vitamin B₁₂ (Fig. 16). The prpDC locus was sequenced for any mutations that might abrogate the activity of the enzymes responsible for 2-methylcitrate / 2-methylisocitrate buildup. However, no mutations were detected at this locus in two independent suppressor mutants. The 5’ coding region of Rv1129c was also sequenced as lesions which altered prpR activity had found to map to the N-terminal domain of the protein in S. enterica (Palacios and Escalante-Semerena, 2004), but no mutations were uncovered. CobB, an enzyme of the cobalamin biosynthetic pathway is a co-regulator of the of prpBCDE operon in S. typhimurium (Tsang et al., 1998). Therfore cobB in MTB will be examined for lesions in ongoing studies.

Preliminary investigations found that the specialised polymerases DnaE2 and DinB1/DinB2 were not involved in the emergence of suppressor mutants under conditions of metabolite stress investigated in this study (Fig. 18). Interestingly, PolI mutants in S. typhimurium are unable to grow on propionate as a carbon source (Rondon et al., 1995) owing to the
production of reactive intermediates during propionate catabolism by the methylcitrate cycle which exert damaging effects on DNA that require PolII editing function during DNA replication. Therefore, in the presence of an alternate propionate oxidizing pathway, selection for mutations which inactivate the methylcitrate cycle is likely to be strong. It has yet to be established whether stress-induced mutagenesis plays any role in facilitating the emergence of suppressor mutants under these conditions.

5.9 Growth on valerate as a carbon source

In this study H37Rv MTB was found to grow poorly on valerate, a C₅ substrate which yields acetyl-CoA and propionyl-CoA subunits in equal proportion. ICL has a considerably higher affinity for isocitrate (Kₘ = 180 µM) than 2-methylisocitrate (Kₘ = 718 µM) (Gould et al., 2006). Very low levels of 2-methylcitrate / 2-methylisocitrate are considered sufficient to arrest growth (Brock, 2005; Horswill et al., 2001) which raises the question as to whether efficient clearance of these metabolites would occur if ICL catabolises propionate (through the methylcitrate cycle) and acetate (through the glyoxylate cycle) simultaneously. The poor growth of H37Rv on valerate (Fig. 19a) is therefore thought to be due to the potentially crippling effects of methylcitrate cycle intermediate buildup, analogous with the delayed growth of Δicl1 in vitamin B₁₂ supplemented propionate (Fig. 16) The compromised growth of H37Rv on valerate raises question as to the physiological relevance of ICL as an MCL given its apparently limited capacity to catalyse both reactions simultaneously. However should MTB encounter only longer chain fatty acids (see section 5.10) and/or cholesterol (Pandey and Sassetti, 2008) in vivo low levels of MCL activity may be sufficient.

The findings on propionate established that the methylmalonyl-CoA pathway can metabolise propionate independently of the methylcitrate and glyoxylate cycles. However, the dual operation of the methylmalonyl pathway and the glyoxylate cycle may be required for optimal growth on valerate. Indeed, the markedly improved growth on valerate in the presence of vitamin B₁₂ suggested the regulated partitioning of propionyl-CoA into the methylmalonyl pathway and acetyl-CoA into the TCA cycle.
The transcriptional data for growth on valerate are consistent with propionate oxidation primarily through the methylmalonyl pathway (Fig. 20). Further downregulation of prpD and icl expression on valerate with vitamin B_{12} relative to propionate with vitamin B_{12} suggests that flux through the methylcitrate and glyoxylate cycle is minimal. This is also consistent with metabolite rather than vitamin B_{12}-mediated regulation of transcription.

The key finding that no differential phenotype could be detected in the H37Rv vs. ΔprpDC for growth on valerate in the presence of 3NP and vitamin B_{12} (Fig. 21a) reinforced the notion that methylmalonyl pathway is the default route for propionyl-CoA oxidation in the presence of vitamin B_{12} and acetyl-CoA (derived from valerate). On valerate with vitamin B_{12}, chemical inhibition of ICL with 3NP (Fig. 21a) did not result in the 2-methylcitrate / 2-methylisocitrate-mediated growth inhibitory effects observed on propionate with vitamin B_{12} (Fig. 17a), consistent with the 2.6-fold lower level of prpD expression in vitamin B_{12}-supplemented valerate compared to propionate. (Fig. 20). Furthermore, the expression data suggested an absence of any significant prpD induction in valerate with vitamin B_{12} relative to 7H9 (Fig. 20). Therefore, these findings suggest that in the presence of an active methylmalonyl pathway, the flux through the methylcitrate cycle is substantially reduced in cultures grown on valerate. This reflects an important facet of propionate regulation at the PCC/MCS junction. The marked upregulation of prpDC genes (Schnappinger et al., 2003) in macrophages may be an indication that vitamin B_{12} is unavailable ex vivo, and may explain the requirement of prpDC for MTB replication in macrophages (Muñoz-Elias et al., 2006).

5.9.1 Anaplerotic role for the methylmalonyl pathway on valerate

Growth of H37Rv on valerate with vitamin B_{12} was unaffected by the presence of 3NP (Fig. 21a). This demonstrates the anaplerotic contribution of the methylmalonyl pathway, as growth on a theoretical mixture of propionate and acetate is supported in the absence of a glyoxylate cycle. The robust growth on valerate with 3NP in the presence of vitamin B_{12} confirms that the generation of energy equivalents by the active TCA cycle satisfies the demand for gluconeogenesis on this fatty acid carbon source. Therefore, the expression data, the absence of putative toxic methylcitrate cycle intermediate accumulation (Fig. 21a) and the dispensability of glyoxylate cycle (Fig. 22) together substantiates the methylmalonyl pathway.
as the preferred route for propionate metabolism and establishes the fundamental role of the
methylmalonyl pathway in anaplerosis.

The finding that the Δicl1 mutant was unable to grow on valerate despite vitamin B_{12}
supplementation was unexpected as it was not consistent with the robust growth observed for
H37Rv on vitamin B_{12}-supplemented valerate in the presence of 3-NP. Singh and Ghosh
predicted approximately 24% flux through the glyoxylate cycle in the presence of 0.1mM 3NP
(Singh and Ghosh, 2006). Therefore, one possible explanation for the lack of growth of the
Δicl1 mutant on vitamin B_{12}-supplemented valerate is that some minimal threshold activity
regulates metabolite flow at the ICL/ICD (glyoxylate cycle/TCA) branch point preventing the
buildup of derivative acetyl-CoA. Acetate inhibition of growth has been shown to arise from
the depletion of intracellular methionine pools with the concomitant accumulation of the toxic
intermediate, homocysteine (Roe et al., 2002a). Alternatively, some baseline activity of ICL
(~24%), while not sufficient to support growth on valerate with 3NP (Fig. 21a), may be
adequate to prevent 2-methylcitrate/2-methylisocitrate buildup from propionyl-CoA that
potentially ‘overflows’ into the methylcitrate cycle on vitamin B_{12} supplemented valerate with
3NP. Three independent lines of evidence support this notion. (1) No anaplerosis by the
glyoxylate cycle is necessary on valerate with vitamin B_{12} when the methylmalonyl pathway
predominates (Fig. 22). Growth on valerate with vitamin B_{12} is orchestrated by the
methylmalonyl pathway in conjunction with the TCA cycle. (2) Extremely low levels of 2-
methylisocitrate are sufficient to inhibit ICD (Brock, 2005; Horswill et al., 2001) and, has
been shown that in response to such inhibition, the specific activity of ICD is increased
(Brock, 2005). (3) ICL and ICD compete for a common substrate (Walsh and Koshland,
1985), so given the rather low affinity of ICL for isocitrate relative to ICD (Holms, 1987), the
residual ICL may, by default, fulfill a dedicated MCL function. Therefore, given the evidence
that there is little requirement for the role of ICL in the glyoxylate cycle and the compensatory
action of ICD to 2-methylcitrate/2-methylisocitrate inhibition, ICL may perform exclusively
as a MCL during on valerate supplemented with vitamin B_{12}.

In an ICL-deficient mutant, clearance of methylcitrate cycle intermediates would be entirely
abrogated. For the reasons outlined above, the resultant flow of isocitrate at the ICL / ICD
junction would be potentially severed. Additionally, in this background, increasing levels of 2-
methylcitrate would target aconitate and citrate synthase (Cheema-Dhadli et al., 1975), manifesting in acetyl-CoA buildup which would further thwart efforts by ICD to maintain isocitrate homeostasis in the absence of ICL function. Moreover, decreasing the generation of energy equivalents in this way would exacerbate energy loss due to uncoupling of the membrane potential by acetate buildup (Baronofsky et al., 1984). This reasoning is supported by the growth on similar fatty acids of other mycobacteria which lack the glyoxylate cycle but maintain an intact methylcitrate cycle by virtue of having a dedicated MCL. For example, a MLS mutant of Mycobacterium sp. strain P101 is able to grow on pristanic acid, which is also oxidised to a propionyl-CoA:acetyl-CoA ratio of 1:1 (Sakai et al., 2004). An Δicl1Δicl2 mutant of MSM is also able to grow on valerate (Muñoz Elías, 2005). Here, the ability of ICL-deficient mycobacterial strains to grow on these fatty acids may be largely due to detoxifying MCL (prpB) activity, which is entirely absent in MTB Δicl1. In keeping with this hypothesis, a ΔprpDCΔicl1 double mutant would be expected to grow in valerate supplemented with vitamin B12.

Finally, understanding the disparity between genetic (knockout) and chemical (3-NP) loss of ICL function is of considerable importance in terms of drug targeting against ICL. By analogy with the relatively invulnerable mycobacterial DNA ligase, whose activity can be reduced by 90% without any effect on survival (Korycka-Machala et al., 2007), it is possible that (incomplete) chemical inhibition of ICL may have little effect on mycobacterial cell viability.

5.10 Growth on heptadecanoate as a carbon source

Markedly improved growth of H37Rv was observed on heptadecanoate (Fig. 23) relative to valerate (Fig. 19a). In this case, oxidation of the propionyl-CoA moiety released from oxidation of the C17 substrate did not appear to cause sufficient buildup of toxic, methylcitrate cycle intermediates believed to account for the retarded growth on valerate. Furthermore, the methylcitrate cycle is dispensable for growth on this odd-chain fatty acid (Fig. 23), contrary to the failure of ΔprpDC to grow on propionate (Fig. 11) and valerate (Fig. 19). Subculturing of ΔprpDC revealed no detectable growth inhibitory effects, which would have suggested propionyl-CoA accumulation over time. Furthermore, growth of ΔprpDC was restored to that of wildtype on the second passage (data not shown). This adaptation suggests that derivative
propionyl-CoA not able to be oxidized as a carbon and energy source, may instead be accommodated into cellular lipids during derivative acetyl-CoA-dependent growth. Here endogenous propionyl-CoA is carboxylated by PCC to methylmalonyl-CoA extender subunits required for methyl-branched fatty acid synthesis (see section 5.11), consistent with the essentiality of AccD5 (Holton et al., 2006), accA3 and accE5 (Sassetti et al., 2003). However, given the poor growth of H37Rv on valerate (Fig. 19a), the amount of intracellular propionyl-CoA diverted into this alternate route appears to be restricted, although variable and subject to differences between strains, as discussed in section 5.11.

Valerate media may be approximated to a carbon mixture of 50% acetate and 50% propionate (1:1) (bearing in mind the addition of 0.05% Tween-80 in the media). Consistent with this, a ΔprpDC mutant of MTB Erdman is unable to grow in media containing either a mixture of 50% acetate and 50% propionate or valerate as the carbon source (Muñoz-Elias et al., 2006). This is contrary to ΔprpC2 of C. glutamicum which is able to grow in media comprising a carbon mixture of 50% propionate and 50% acetate (Plassmeier et al., 2007), despite being unable to grow solely on propionate (Claes et al., 2002). Varying carbon balances in media with decreasing concentrations of propionate has been shown to relieve growth inhibition (Brock and Buckel, 2004). In this study growth of ΔprpDC on heptadecanoate (Fig. 23), which compares to a carbon mixture of 85% acetate and 15% propionate (7:1), but not on valerate (Fig. 19a) indicates that MTB in the absence of propionate oxidizing pathways is able to grow on longer odd-chain fatty acids provided propionate is sufficiently diluted relative to acetate. Growth on heptadecanoate in the absence of any propionate oxidizing pathways has also been reported for the actinomycete A. erythreum (Reeves et al., 2004). Should growth on heptadecanoate most accurately reflect the in vivo environment it may explain the dispensability of prpDC in vivo (Muñoz-Elias et al., 2006).

In A. fumigatus a ratio between acetyl-CoA and propionyl-CoA greater than 4 (specifically 4.8:1) has no adverse effect on growth of wild type or mutant strains unable to oxidise propionate (Ibrahim-Granet et al., 2008). Similarly, this study established that for a ratio of 7:1 acetate to propionate (for C17), MTB experiences no apparent propionyl-CoA toxicity. From this it may be may assumed that the endogenous ratio of acetyl-CoA:propionyl-CoA generated by the oxidation of odd-chain amino acid and/or fatty acid turnover is ≥ 7 given that
a ΔprpDC mutant of MTB shows no growth deficit on 7H9 media, as is the case for *C. glutamicum* where the methycitrate cycle has a nominated ‘housekeeping function’ (Plassmeier *et al.*, 2007).

The anaplerotic role of the methylmalonyl pathway in MTB on odd-chain fatty acids longer than valerate has yet to be demonstrated. Interestingly, growth is possible on nonanoic acid in *Pseudomonas putida* lacking isocitrate lyase (Nieder and Shapiro, 1975). Growth on this odd-chain fatty acid was ascribed to the organism’s ability to form and incorporate propionyl-CoA, although no pathway was implicated. In *C. glutamicum* the activity of the glyoxylate cycle significantly diminishes on addition of propionate to acetate media which has been speculated to be due to the anaplerotic reaction catalysed by PCA which uses the pyruvate produced by the methycitrate cycle. In MTB, the findings from this study suggest that as the ratio of derivative propionyl-CoA decreases relative to acetyl-CoA, the anaplerotic contribution of an active methylmalonyl pathway would diminish, as would its ability to support growth on odd-chain fatty acids of increasing length in the absence of a functional glyoxylate cycle (Fig. 26).
Fig. 26 Proposed model detailing the variable anaplerotic contribution of the methylmalonyl pathway relative to the glyoxylate cycle on odd-chain fatty acids.
As the relative ratio of propionate (C₃) to acetate (C₂) increases along the gradient of decreasing length odd-chain fatty acids, the anaplerotic contribution of the methylmalonyl pathway is proposed to become more pronounced, provided the vitamin B₁₂-derived cofactor requirements are met. In the absence of relatively high concentrations of propionate relative to acetate, the glyoxylate cycle may predominate. The direction of decreasing magnitude is illustrated by the narrowing triangular neck.

In accordance with this model, two possible explanations for the attenuated phenotype of MTB infecting macrophages with added 3NP (Muñoz-Elias and McKinney, 2005) are offered. Either vitamin B₁₂ supplies may be limiting ex vivo or alternately insufficient propionyl-CoA is metabolised through the anaplerotic methylmalonyl pathway to afford growth in the absence of the glyoxylate cycle.

Altering intracellular pathway fluxes by vitamin B₁₂ supplementation may also increase resistance to ICL inhibitors, as observed for 3NP in valerate with vitamin B₁₂. The action of the methylmalonyl pathway may also alter MTB susceptibility to other classes of antibiotics. It has been recently shown that several antibiotics exert a bactericidal effect through the common induction of oxidative stress independent of drug-target interaction (Dwyer et al., 2007; Kohanski et al., 2007). This oxidative damage pathway resulting in cellular death is triggered by NADH depletion and superoxide formation by hyperactivation of the electron transport chain. These events promote destabilization of iron-sulfur clusters, stimulation of the Fenton reaction and cell death. Diminution of the TCA cycle decreases NADH production and consequently decreases superoxide generation. Modulation of the cellular redox environment by the TCA cycle and its established link with ROS homeostasis (Mailloux et al., 2007; Ogasawara et al., 2007) can be exploited by organisms for increased resistance to oxidative stress (Husain et al., 2008; Mailloux et al., 2007) or such bactericidal antibiotics (Kohanski et al., 2007). Therefore, the MICs of anti-tubercular drugs may vary as the available pool of NADH generated during the TCA cycle is increased when the methylmalonyl pathway is the major propionate oxidizing route. This is consistent with carbon source utilization influencing the intracellular redox state (Singh et al., 2007a). Accordingly, susceptibility to oxidative
stress in cultures grown on odd- and even-chain carbon sources with and without vitamin B<sub>12</sub> should be evaluated.

### 5.11 Propionyl-CoA incorporation into virulence lipids relieves toxicity on valerate

The ability of MTB to grow on valerate appears to be subject to inter-strain variation. Unlike the data reported from other studies, the strain of H37Rv used in this study grew relatively poorly on valerate (Chang et al., 2007; Muñoz-Elías et al., 2006). Furthermore, Muñoz-Elías showed that ICL1-dependent growth of the Δicl2 mutant of MTB Erdman on valerate does not phenocopy the H37Rv strain employed in this study, despite possessing only a single functional ICL (Muñoz Elías, 2005). Instead, growth on valerate was similar to the parental Erdman strain (Muñoz-Elias et al., 2006).

This perplexing observation suggested the possibility of an alternate route for ‘diffusion’ of propionyl-CoA, evident in Erdman Δicl2 on valerate and, to a lesser, extent in H37Rv on heptadecanoate. If one reconciles this notion with the observation that in the absence of a methylcitrate cycle, H37Rv fails to grow on valerate but not on heptadecanoate, the siphoning of propionyl-CoA into methylmalonyl-CoA -and hence into lipid synthesis, in a concentration dependent manner -emerges as a likely explanation for the differences observed. The H37Rv strain used in this study is a poor producer of PDIM (Kana et al., 2008) unlike the Erdman strain, which produces high quantities of this lipid (Prof. J. McKinney, personal comm.). Therefore, production of PDIM can drain the methylmalonyl-CoA pool and hence, provide a sink for intracellular propionyl-CoA. Significant shedding of PDIM into culture media containing 0.05% Tween (Camacho et al., 2001) supports the notion of a propionyl-CoA ‘sink’. Jain et al. observed a significant increase in PDIM for MTB grown in propionate and valerate (Jain et al., 2007) and proposed that this phenotype was the direct result of increasing intracellular levels of the biosynthetic precursor of polyketide lipids, namely, methylmalonyl-CoA. Furthermore, propionyl-CoA released from oxidation of the cholesterol side chain is assimilated into PDIM in activated macrophages (Pandey and Sassetti, 2008). Consistent with the observations is the finding that accD5 is strongly upregulated in MTB grown in IFN-γ.
activated macrophages vs. resting macrophages (Rachman et al., 2006a) which may explain why the \( \Delta prpDC \) mutant of MTB Erdman is severely impaired in resting vs. activated macrophages (Muñoz-Elias et al., 2006). In this respect, the virulence attributes of PDIM extend beyond its antigenic properties to encompass a broader role in propionate metabolism. The markedly improved growth on valerate of a PDIM-producing isolate of H37Rv compared to the isolate employed in this study (Fig. 24) was consistent with this notion. However, this may be an over-simplified explanation as excess methylmalonyl-CoA has been shown to be diverted into hydroxyacids and further incorporated into sulpholipids in a PDIM mutant (Sirakova et al., 2003). Pks2 incorporation of methylbranches (using methylmalonyl-CoA) into virulence lipid SL-1 (Sirakova et al., 2001) was also shown to increase dramatically with increasing concentrations of propionate resulting in a greater abundance and mass of SL-1 (Jain et al., 2007). Perturbations in the methylmalonyl-CoA supply would likely have pleiotropic effects as this substrate is necessary for the biosynthesis of multiple methyl-branched fatty acids including phthioceranic, hydroxyphthioceranic, mycosanoic, and mycolipenic acids, which form glycolipids PDIM, SL-1, diacyl trehaloses (DAT), and pentaacyl trehalose (PAT) (Jackson et al., 2007; Minnikin et al., 2002).

This raises the question as to what extent cell envelope remodeling may occur in the H37Rv strain employed in this study to accommodate a propionyl-CoA sink when grown on valerate and heptadecanoate. Clearly, the complex regulation of methyl-branched fatty acids is subject to strain variation and is not simply dictated by the availability of methylmalonyl-CoA. Further insight will be gained from ongoing investigations into the role of MCM in secondary metabolism of polyketide synthesis.

Diverting propionyl-CoA metabolites into virulence lipids this way may regulate flux primarily through the methylmalonyl pathway. This study has shown that an active MCM enzyme (and not vitamin B_{12} \textit{per se}) decreases flux through the methylcitrate cycle. It is possible, therefore, that an active MCM would reduce the methylmalonyl-CoA metabolite pool, in response to which flux through the methylcitrate cycle may be ‘turned-down’ to compensate for flow through the methylmalonyl branch to sustain methylmalonyl-CoA homeostasis. This proposal is substantiated by recent studies which linked \textit{mutAB} overexpression in cultures grown in 7H9 to a 30% and 80% drop in PDIM and SL-1
production respectively (Jain et al., 2007), suggesting methylmalonyl-CoA sequestration by MCM despite the absence of vitamin B_{12} in the 7H9 growth media. Such a proposed coherence of reaction fluxes in the network is described for metabolites with a low degree of connectivity, such as methylmalonyl-CoA (Samal et al., 2006).

5.11.1 Maintaining methylmalonyl-CoA homeostasis in MTB

Propionyl-CoA as the terminal product of β-oxidation and as a precursor for polyketide lipid biosynthesis, including SL-1 and PDIM, provides an intimate link between central carbon catabolism and virulence (Brown et al., 2008). Mutants deficient in multimethyl-branched fatty acids such as PDIMs (Cox et al., 1999; Sirakova et al., 2003), related but structurally distinct phenolic glycolipids (Reed et al., 2004) and SL-1 (Converse et al., 2003), are attenuated in mice although the mechanism by which the absence of these virulence lipids causes attenuation is unclear. Presumably, they are involved in immune modulation as they form a significant portion of the noncovalently linked lipids in the mycobacterial outer membrane leaflet (Zuber et al., 2008). However, another mode of action of this family of virulence factors may be in maintaining the physical properties of the cell wall such as reducing cell wall permeability (Camacho et al., 2001) and protecting against bacteriocidal activity of reactive nitrogen intermediates released by activated macrophages (Rousseau et al., 2004). MTB may have adopted specific mechanisms to ensure that a biosynthetic deficiency of such virulence lipids is limited.

An increase in PDIM synthesis is reported for bacteria growing within host tissue (Jain et al., 2007). Significantly, Jain et al. (2007) concluded that such virulence polyketide anabolism is directly regulated by a metabolic shift to growth on odd-chain fatty acids in vivo, given that PDIM and SL-1 production was stimulated during growth on propionate and valerate, but not on acetate. However the possibility that bacteria exploit the reverse succinyl-CoA →methylmalonyl-CoA mutase reaction as an adaptive response to insufficient propionyl-CoA precursor in vivo cannot be excluded and requires further investigation. Consistent with such a proposal, methylmalonyl-CoA production for PDIM synthesis may have increased as a result of MCM activity in host tissue, assuming the B_{12}-cofactor is accessible in vivo. Similarly,
should vitamin B$_{12}$ be supplied to acetate grown cultures, a similar increase in PDIM synthesis may occur by virtue of raised levels of intracellular glyoxylate cycle-derived succinate diverted to methylmalonyl-CoA through MCM. Comparisons of lipid profiles on acetate or butyrate with and without vitamin B$_{12}$ would offer insight into the contributing role of MCM on even-chain fatty acids.

*In vitro* (outside the living host), the carboxylation of propionyl-CoA by PCCase (accD5/accA3/accE5) is an essential reaction (Holton *et al.*, 2006; Sassetti *et al.*, 2003) supporting the notion that under standard culturing conditions, only this pathway contributes to the generation of methylmalonyl-CoA. However this reaction may be conditionally essential subject to the availability of vitamin B$_{12}$ on succinate and/or acetate. In this instance, an *accD5* mutant of MTB might be viable for growth on succinate and/or acetate media supplemented with vitamin B$_{12}$, as demonstrated in this study for Δ*prpDC* on propionate supplemented with vitamin B$_{12}$. Conversely, a *mutAB* mutant may be attenuated for growth *in vivo* should methylmalonyl-CoA be generated via this route in the human host.

Targeting virulence has been proposed as a new approach in antimicrobial development (Clatworthy *et al.*, 2007). The increased pathology of MTB clinical isolates has been attributed to differences in the surface exposed lipids (Manca *et al.*, 2001). In this context, targeting genes which perturb methylmalonyl-CoA pools presents an attractive option.

### 5.12 A role for MCM in acetate assimilation

Aside from the glyoxylate cycle MCM plays a role in all other described alternate pathways for acetate assimilation. Notably these alternate pathways require complex carbon skeletal rearrangements involving cobalamin as a cofactor. These are the glyoxylate regeneration cycle for *M. extorquens* (Korotkova *et al.*, 2002; Korotkova *et al.*, 2005), the citramalate cycle (see Fig. 25) in *Rhodospirillum rubrum* (Ivanovsky *et al.*, 1997) and the ethylmalonyl-CoA pathway of *R. sphaeroides* (Alber *et al.*, 2006; Erb *et al.*, 2007; Erb *et al.*, 2008). These alternate anaplerotic reactions are not restricted to ICL-deficient bacteria. Alternate acetyl-CoA assimilation has been shown to operate independently of the glyoxylate cycle in *Streptomyces collinus* grown on acetate without Tween (Han and Reynolds, 1997). As
mentioned above, interrogation of the MTB genome for alternative acetate assimilation pathways is complicated as the enzyme activities for all the steps in these pathways have yet to be resolved. Both the ethylmalonyl-CoA pathway and the proposed citramalate cycle in MTB (Fig. 25) involve a complex series of carboxylation and rearrangement reactions involving the newly discovered enzyme L-malyl-CoA lyase/β-methylmalyl-CoA lyase (see Fig. 25) (Meister et al., 2005), as well as enzymes of propionate metabolism (MCM) to yield succinate. However, preliminary investigations revealed that chemical inhibition of ICL using 3NP resulted in no growth of MTB on acetate supplemented with vitamin B₁₂. Similarly, MSM, which is capable of producing sufficient vitamin B₁₂ to support its vitamin B₁₂-dependent enzymes (Karasseva et al., 1977) (D. Warner & S. Dawes, unpublished observations) cannot grow on acetate in the absence of ICL (Muñoz Elías, 2005).

Therefore, in MTB, the glyoxylate cycle seems to be the only pathway for assimilating acetate in vitro. However the possible upregulation of alternate acetate pathway genes in vivo cannot be excluded. It may therefore be argued that the in vivo essentiality of ICL in mice might be attributed, at least in part, to insufficient B₁₂-coenzyme.

The carbon rearrangement of methylmalonyl-CoA to succinyl-CoA by coenzyme vitamin B₁₂ dependent MCM is also an integral part of autotrophic carbon dioxide assimilation routes (Berg et al., 2007; Herter et al., 2002a). In the described 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway (Berg et al., 2007; Strauss and Fuchs, 1993), CO₂ fixation by the methylmalonyl pathway enzyme, PCC and acetate assimilation cycles are intimately linked through a series of common enzymes, with both pathways requiring MCM. This raises the intriguing question as to whether similar cycles may be operational in MTB, provided the cofactor requirements are met. An alternate strategy for C₂ assimilation may afford some advantage to MTB under nitrosative stress where enzymes of the glyoxylate cycle, aconitase and MLS are inhibited (Rhee et al., 2005). The possibility that the MCM reaction may be involved in such other metabolic pathways in MTB remains to be addressed.
CONCLUDING REMARKS

MTB as an obligate pathogen needs to survive in an increasingly complex and heterogeneous environment in the human lung as disease progresses. This requirement is illustrated by the rich and diverse set of genes (Cole et al., 1998) which this organism possesses to optimise survival in the numerous cellular niches serving as potential habitats within the human host (Hernandez-Pando et al., 2000; Neyrolles et al., 2006; van der Wel et al., 2007). The metabolic adaptability of this organism is a key component of virulence.

Given the central role occupied by propionyl-CoA in cellular metabolism as both a terminal product of β-oxidation of odd- and branched-chain fatty acids and a precursor in several lipid biosynthetic pathways (Kolattukudy et al., 1997), this study has focused on characterizing the pathways for propionate metabolism as an important facet of MTB physiology. Host cholesterol is avidly exploited as a carbon source by this organism (Mohn et al., 2008; Pandey and Sassetti, 2008; Senaratne et al., 2008; Van der Geize et al., 2007) potentially releasing copious amounts of derivative propionyl-CoA during oxidation of this sterol. MTB possesses alternate pathways for oxidizing this high-energy metabolite owing to the toxic side-effects of derivative propionyl-CoA accumulation (Brock and Buckel, 2004).

Marked up-regulation of prpC and prpD in the macrophage (Schnappinger et al., 2003) revealed a methylcitrate cycle in MTB, and is consistent with the propionate-induced activity of MCS in vitro (Muñoz-Elias et al., 2006). However, a prpDC-deficient of MTB is not attenuated for growth in vivo (Muñoz-Elias et al., 2006) which signifies the presence of alternate buffering pathway(s). As methylmalonyl-CoA is a precursor of essential methyl-branched lipids (Camacho et al., 2001; Minnikin et al., 2002; Rainwater and Kolattukudy, 1983), propionyl-CoA may be diverted into anabolic pathways for cell wall synthesis and maintenance in vivo. This is consistent with the growth on a C₁₇ fatty acid of the ΔprpDC mutant of the H37Rv strain of MTB observed in this study. Alternatively, propionyl-CoA may be oxidised to succinyl-CoA by the vitamin B₁₂-dependent methylmalonyl pathway. In this study, the functionality of this anaplerotic pathway subject to supplementation of media with
vitamin B\textsubscript{12} was demonstrated, reinforcing the potential importance of vitamin B\textsubscript{12} in mycobacterial pathogenesis.

Pathway(s) preference by MTB for propionate oxidation \textit{in vivo} is likely to be balanced by the availability of vitamin B\textsubscript{12} and the demands for TCA cycle intermediates and methyl-branched lipids as a function of stage of infection and microenvironment encountered by the pathogen within the host. For example, although ICL is essential during acute infection (Muñoz-Elias and McKinney, 2005), it is downregulated in end-stage granulomas concomitant with the upregulation of \textit{meaB} (Rachman \textit{et al.}, 2006b). Therefore, the role of the MCM pathway \textit{in vivo} has been prioritized for ongoing and future investigation. Important areas for further study include characterising different MTB strains by their ability to synthesize methyl-branched polyketides as a means of detoxifying derivative propionyl-CoA by monitoring growth of $\Delta$prpDC on odd-chain fatty acids of chain length between the two extremes employed in this study (\textit{i.e.} $>5$ and $<17$). Furthermore, validation of the model proposed in this study (Fig. 26) requires that anaplerosis by the methylmalonyl pathway on odd-chain fatty acids of chain length greater than five be assessed as a function of growth independent of the glyoxylate cycle. Future work will also focus on the construction of $\Delta$mutAB$\Delta$prpDC and $\Delta$prpDC$\Delta$icl1$\Delta$icl2 mutants of MTB Erdman and assessment of their ability to grow and persist in macrophages and in mice to establish the relevance of propionate metabolism in MTB pathogenesis. A possible essential role for MCM in propionate and/or acetate metabolism and/or polyketide synthesis during the chronic stage infection will be investigated in parallel by assessing the virulence of a $\Delta$mutAB mutant of MTB Erdman. Such studies should provide key insights into the availability of vitamin B\textsubscript{12} cofactor \textit{in vivo}. 


# APPENDICES

## Appendix 1: List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Abbreviated term/phrase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>Acyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACN</td>
<td>Aconitase</td>
</tr>
<tr>
<td>AdoCba</td>
<td>Adenosylcobinamide</td>
</tr>
<tr>
<td>AdoCbl</td>
<td>Adenosylcobalamin</td>
</tr>
<tr>
<td>AHTc</td>
<td>Anhydrotetracycline</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>aph</td>
<td>Gene encoding aminoglycoside phosphotransferase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATR</td>
<td>Adenosyl transferase</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched chain amino acids</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacilli Calmette-Guérin</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CIT</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>CMS</td>
<td>Citramalate synthase</td>
</tr>
<tr>
<td>CNCbl</td>
<td>Cyanocobalamin (vitamin B(_{12}))</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>CRP</td>
<td>cAMP receptor protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMB</td>
<td>Dimethylbenzimidazole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DOTS</td>
<td>Directly observed therapy, short-course</td>
</tr>
<tr>
<td>EHR</td>
<td>Enduring Hypoxic Response</td>
</tr>
<tr>
<td>EMB</td>
<td>Ethambutanol</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FNR</td>
<td>Fumurate-nitrate regulator</td>
</tr>
<tr>
<td>FUM</td>
<td>Fumurase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Hyg</td>
<td>Hygromycin B</td>
</tr>
<tr>
<td>Hyg</td>
<td>Gene conferring resistance to hygromycinB</td>
</tr>
<tr>
<td>ICD</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>ICL</td>
<td>Isocitrate lyase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl thio-β-D-galactoside</td>
</tr>
<tr>
<td>IRIS</td>
<td>Immune reconstitution inflammatory syndrome</td>
</tr>
<tr>
<td>LTBI</td>
<td>Latent TB infection</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>2KG</td>
<td>α-Ketoglutarate</td>
</tr>
<tr>
<td>KGD</td>
<td>α-Ketoglutarate decarboxylase</td>
</tr>
<tr>
<td>Km</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>K_m</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>lacZ</td>
<td>Gene encoding β-galactosidase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MCD</td>
<td>Methylcitrate dehydratase</td>
</tr>
<tr>
<td>MCL</td>
<td>Methylcitrate lyase</td>
</tr>
<tr>
<td>MCM</td>
<td>Methylmalonyl-CoA mutase</td>
</tr>
<tr>
<td>MCR</td>
<td>α-Methylacyl-CoA racemase</td>
</tr>
<tr>
<td>MCS</td>
<td>Methylcitrate synthase</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistant</td>
</tr>
<tr>
<td>MEZ</td>
<td>Malic enzyme</td>
</tr>
<tr>
<td>MLS</td>
<td>Malate synthase</td>
</tr>
<tr>
<td>MMEE</td>
<td>Methylmalonyl-CoA epimerase</td>
</tr>
<tr>
<td>MQO</td>
<td>Malate:quinine oxidoreductase</td>
</tr>
<tr>
<td>MSM</td>
<td><em>Mycobacterium smegmatis</em></td>
</tr>
<tr>
<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>NDH</td>
<td>NADH dehydrogenase</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitritriacetic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>3-NP</td>
<td>3-Nitropropionate</td>
</tr>
<tr>
<td>OADC</td>
<td>Albumin-dextrose complex supplement with oleic acid for Middlebrook 7H9</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at 600 nanometre wavelength</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCA</td>
<td>Pyruvate carboxylase</td>
</tr>
<tr>
<td>PCC</td>
<td>Propionyl-CoA carboxylase</td>
</tr>
<tr>
<td>PCK</td>
<td>PEP carboxykinase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PDHC</td>
<td>Pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>PDIM</td>
<td>Phthiocerol dimycocerates</td>
</tr>
<tr>
<td>PDP</td>
<td>Petal death protein</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPM</td>
<td>PEP mutase</td>
</tr>
<tr>
<td>PKS</td>
<td>Polyketide synthase</td>
</tr>
<tr>
<td>PMF</td>
<td>Proton motive force</td>
</tr>
<tr>
<td>PPC</td>
<td>PEP carboxylase</td>
</tr>
<tr>
<td>PPS</td>
<td>PEP synthase</td>
</tr>
<tr>
<td>PYK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PZA</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>RBS</td>
<td>Ribosome binding site</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription/transcriptase</td>
</tr>
<tr>
<td>sacB</td>
<td>Gene encoding levansucrase</td>
</tr>
<tr>
<td>SCS</td>
<td>Succinate synthase</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acids</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>SL</td>
<td>Sulphlipids</td>
</tr>
<tr>
<td>SSADH</td>
<td>Succinic semialdehyde dehydrogenase</td>
</tr>
<tr>
<td>TA</td>
<td>Toxin-antitoxin</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tween</td>
<td>Polyoxyethylene sorbitan monooleate</td>
</tr>
<tr>
<td>XDR</td>
<td>Extreme drug resistance</td>
</tr>
</tbody>
</table>
7.2 Appendix 2: Culture Media

All media was made up to a final volume of 1 litre deionised water, and sterilized by autoclaving at 121°C for 15 minutes.

Luria-Bertani broth (LB)
10g tryptone powder; 5g yeast extract; 10g sodium chloride

Middlebrook-OADC (7H9-OADC)
4.7g Middlebrook 7H9 broth base; 2ml glycerol
100ml OADC supplement and 0.05% Tween 80 added after autoclaving.

Middlebrook-OADC plates (7H10-OADC)
19g Middlebrook 7H10 agar powder; 5ml glycerol
100ml OADC supplement and 0.05% Tween 80 added after autoclaving.

Propionate media
4.7g Middlebrook 7H9 broth base
0.5% Albumin
0.085% NaCl
0.05% Tween-80
0.1% or 0.2% sodium propionate
10 µg/ml vitamin B₁₂ (optional)
Valerate media

4.7g Middlebrook 7H9 broth base
0.5% Albumin
0.085% NaCl
0.05% Tween-80
0.1% Valeric acid

pH of the valeric acid-containing medium was adjusted to 6.8 with 10M NaOH.

10 µg/ml vitamin B<sub>12</sub> (optional)

Heptadecanoic acid

4.7g Middlebrook 7H9 broth base
0.5% Albumin
0.085% NaCl
0.05% Tween-80
0.007% Heptadecanoic acid (from pre-warmed 0.2% stock solution)

10 µg/ml vitamin B<sub>12</sub> (optional)
References


Fig.S. 1 Vector Maps
Maps of vectors used in this study i) mutAB with an internal deletion of 2342bp carried on the suicide vector p2ΔmutAB17 (Parish and Stoker, 2000); ii) Full length mutAB used for complementation carried on the suicide vector p2mutAB17 (Parish and Stoker, 2000); iii) Rv1998c placed under the control of the tet-inducible promoter, P\textsubscript{myc1}tetO on the replicating vector pSE100::Rv1998c (Guo \textit{et al.}, 2007); iv) P\textsubscript{myc1}tetO::Rv1998c and the tet-repressor, P\textsubscript{myc1}::tetR expressed together on the integrating vector pMC1m::P\textsubscript{myc1}tetORv1998c (Kaps \textit{et al.}, 2001).
Fig.S. 2 Southern blots

A. Southern blot for prpDC allele.
For the Southern blot analysis (right), genomic DNA from the wild type, ΔprpDC, ΔmutAΔprpDC, ΔmutAB::mutABΔprpDC and Δicl1::hyg was digested with BglII which cuts on either side of the deleted region to produce a 5309-bp fragment from H37Rv and Δicl1::hyg and a 2649-bp fragment from ΔprpDC, ΔmutAΔprpDC and ΔmutAB::mutABΔprpDC which were detected using a 1073bp PCR-generated probe.

B. Southern blot for icl1 allele
For the Southern blot analysis (left), genomic DNA from the wild type and Δicl1::hyg was digested with SalI which cuts on either side of the deleted region to produce a 1072-bp fragment from H37Rv and a 1770-bp fragment from Δicl1::hyg which were detected using a 787bp PCR-generated probe.
<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H37Rv</td>
<td>Virulent reference laboratory strain; ATCC 25618</td>
<td>London School of Hygiene and Tropical Medicine</td>
</tr>
<tr>
<td>ΔmutAB</td>
<td><em>mutAB</em> deletion mutant of H37Rv, lacking 2342 bp internal <em>AscI</em> / <em>BglII</em> fragment</td>
<td>This study</td>
</tr>
<tr>
<td>ΔprpDC</td>
<td><em>prpDC</em> deletion mutant of H37Rv, lacking the 2660 bp region from the start codon of <em>prpD</em> to the stop codon of <em>prpC</em> (Munoz-Elias et al., 2006)</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmutAB::mutAB</td>
<td>Reversion mutant of ΔmutAB in which the wild type mutAB allele was restored by knock-in mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>ΔprpDC::prpDC</td>
<td><em>prpDC</em> mutant carrying a 3115 bp gene region containing the <em>prpDC</em> locus in pPRPDC (Munoz-Elias et al., 2006) integrated at the <em>attB</em> locus; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmutAB ΔprpDC</td>
<td><em>mutAB</em> and <em>prpDC</em> double deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmutAB::mutAB</td>
<td>Double mutant complemented</td>
<td>This study</td>
</tr>
<tr>
<td>ΔprpDC::prpDC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δicl1::hyg</td>
<td><em>Icl1</em> deletion mutant of H37Rv, carrying Δicl1::hyg allele lacking codons for amino acids 65-290 of the 428 amino acid ICL1 protein; Hyg&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Δicl1::hyg::icl1</td>
<td><em>icl1</em> mutant carrying a 1.6kb gene region containing the <em>icl1</em> locus in pICL11 (Muñoz-Elias and McKinney, 2005) as an episomal vector; Hyg&lt;sup&gt;R&lt;/sup&gt; Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>H37Rv::P&lt;sub&gt;myc&lt;/sub&gt;1tetO 1998c::pMC1s</td>
<td>Wild type strain carrying episomal pSE100::Rv1998c plasmid and pMC1s integrated at <em>attB</em> locus</td>
<td>This study</td>
</tr>
<tr>
<td>Δicl1::hyg::P&lt;sub&gt;myc&lt;/sub&gt;1tetO 1998c::pMC1m</td>
<td>icl1 deletion mutant carrying Δicl1::hyg allele with vector pMC1m:: P&lt;sub&gt;myc&lt;/sub&gt;1tetO Rv1998c integrated at <em>attB</em> locus</td>
<td>This study</td>
</tr>
<tr>
<td>H37Rv::pSE100 with pMC1s</td>
<td>Wild type strain carrying episomal pSE100 plasmid and pMC1s integrated at <em>attB</em> locus.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM3Z(+)/f</td>
<td>Cloning vector; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>p2NIL</td>
<td>Cloning vector; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Parish and Stoker, 2000)</td>
</tr>
<tr>
<td>pGOAL17</td>
<td>Plasmid carrying <em>lacZ</em> and <em>sacB</em> genes as a <em>PaeI</em> cassette; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Parish and Stoker, 2000)</td>
</tr>
<tr>
<td>p2mutAB</td>
<td>p2NIL carrying 7760 bp <em>EcoRI</em> fragment excised from the H37Rv cosmid Rv58 (Brosch et al., 1998) and containing the <em>mutAB</em> genes plus 1431 bp of 3'- and 2228 bp of 5'-flanking chromosomal sequence; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>p2ΔmutAB</td>
<td>p2NIL carrying ΔmutAB allele generated by digestion of p2mutAB with <em>AscI</em> and <em>BglII</em> and re-ligation to eliminate 2342 bp of sequence within the <em>mutAB</em> region; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>p2ΔmutAB17</td>
<td>ΔmutAB knockout vector – p2ΔmutAB containing <em>PacI</em> cassette from pGOAL17; Km&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>p2mutAB17</td>
<td><em>mutAB</em> knock-in vector – p2mutAB containing <em>PacI</em> cassette from pGOAL17; Km&lt;sup&gt;R&lt;/sup&gt; Suc&lt;sup&gt;S&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM1998c</td>
<td>Derivative of pGEM3Z(+)/f carrying 3640bp <em>SphI</em> fragment excised from the H37Rv cosmid Rv175 (Brosch et al., 1998) and containing the 777bp Rv1998c gene; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>p2Rv1998c</td>
<td>Derivative of p2NIL carrying 3670bp <em>BamHI-HindIII</em> fragment excised from pGEM1998c and containing Rv1998c; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>p2ΔRv1998c</td>
<td>p2NIL carrying ΔRv1998c allele generated by digestion of p2Rv1998c with NarI and re-ligation to eliminate 312 bp of sequence within the Rv1998c region; Km$^R$</td>
<td>This study</td>
</tr>
<tr>
<td>p2ΔRv1998c17</td>
<td>ΔRv1998c knockout vector – p2ΔRv1998c containing PacI cassette from pGOAL17; Km$^R$ Suc$^S$</td>
<td>This study</td>
</tr>
<tr>
<td>pAU100</td>
<td>pJG1111 carrying ΔprpDC allele – fusion of 1kb PCR products upstream and downstream of prpDC eliminating 2660bp prpDC coding sequence; Km$^R$ Hyg$^R$</td>
<td>(Munoz-Elias et al., 2006)</td>
</tr>
<tr>
<td>pPRPDC</td>
<td>pMV306 (Stover et al., 1991) carrying a 3115 bp EcoRI / NcoI fragment containing prpDC; Km$^R$</td>
<td>(Munoz-Elias et al., 2006)</td>
</tr>
<tr>
<td>pJM056-1</td>
<td>pYUB631 (Pavelka and Jacobs, 1999) carrying a 2.7kb BamHI-ClaI fragment containing icl1 had 685bp internal XhoI fragment deleted and replaced with hyg cassette (McKinney et al., 2000); Hyg$^R$ Km$^R$ Suc$^S$</td>
<td>(McKinney et al., 2000)</td>
</tr>
<tr>
<td>pICL1</td>
<td>pEM2634 (Stover et al., 1991) carrying a 1.6kb SpeI fragment containing icl1; Sm$^R$ (Muñoz-Elias and McKinney, 2005)</td>
<td>(Muñoz-Elias and McKinney, 2005)</td>
</tr>
<tr>
<td>pSE100</td>
<td>pMS2 derivative containing P$_{myc1}$tetO</td>
<td>(Guo et al., 2007)</td>
</tr>
<tr>
<td>pMC1s</td>
<td>pMV306Km derivative, Km$^R$, $p_{myc1}$tetR</td>
<td>(Ehrt et al., 2005)</td>
</tr>
<tr>
<td>pMC1m</td>
<td>pMV306Km derivative, Km$^R$, $p_{myc1}$tetR</td>
<td>(Kaps et al., 2001)</td>
</tr>
<tr>
<td>pSE100::Rv1998c</td>
<td>Derivative of pMS2 with Rv1998c under the control of P$_{myc1}$tetO</td>
<td>This study</td>
</tr>
<tr>
<td>pMC1m::P$_{myc1}$tetO</td>
<td>Derivative of pMS2 with Rv1998c under the control of P$<em>{myc1}$tetO and tetR under the control of P$</em>{imyc}$</td>
<td>This study</td>
</tr>
<tr>
<td>pQE30xa</td>
<td>Expression vector; Amp$^R$</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQE30::prpB</td>
<td>pQE30 carrying prpB from E. coli</td>
<td>(Brock et al., 2001)</td>
</tr>
<tr>
<td>pQE30xa::Rv1998c</td>
<td>pQE30xa (Qiagen) carrying the 783bp BamHI / HindIII fragment containing Rv1998c fused in frame with a 6x His-tag under the control of P$_T5$lacO; Amp$^R$</td>
<td>This study</td>
</tr>
</tbody>
</table>
### Table S. 2 Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)^a</th>
<th>Application</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MutA-F</td>
<td>GGGGTACCGAAGCAAGCCCAAGCA</td>
<td>Forward primer used for PCR genotyping of mutAB and ∆mutAB alleles</td>
<td>2084 bp amplicon generated from the wild type mutAB allele using MutA-F and MutA-R primers</td>
</tr>
<tr>
<td>MutA-R</td>
<td>GGACTAGTCAATGCCTTCCGGCGT</td>
<td>Reverse primer used for PCR-based genotyping of the mutAB allele</td>
<td></td>
</tr>
<tr>
<td>MutB-R</td>
<td>GGAAGCTTGCAGGGCGCTGGCGA</td>
<td>Reverse primer used for PCR-based genotyping of the ∆mutAB allele</td>
<td>1933 bp amplicon generated from the ∆mutAB allele using MutA-F and MutB-R primers</td>
</tr>
<tr>
<td>MutAB-F</td>
<td>AGATCCGTACCATTCCGGG</td>
<td>Forward and reverse primer pair used to generate the DNA probe used for genotypic confirmation of the ∆mutAB mutant by Southern blot analysis</td>
<td>904 bp amplicon spanning from the 3’-end of mutB to within Rv1495 (see Fig. S1 in the Supplementary Information)</td>
</tr>
<tr>
<td>MutAB-R</td>
<td>TTGTGGAGACGATGAGCCA</td>
<td>Reverse primer used for PCR genotyping of the prpDC and ∆prpDC alleles</td>
<td>1073 bp amplicon generated from wild type prpDC allele using PrpDC-F and PrpDC-R</td>
</tr>
<tr>
<td>PrpDC-F</td>
<td>GGGGGCTGCTCTGCGGCACCGGTG</td>
<td>Forward primer used for PCR-based genotyping of the prpDC and ∆prpDC alleles</td>
<td></td>
</tr>
<tr>
<td>PrpDC-R</td>
<td>GGGGGATCTTGTAGGCCATGTGCTC</td>
<td>Reverse primer used for PCR genotyping of prpDC allele</td>
<td></td>
</tr>
<tr>
<td>PrpDC-R2</td>
<td>GGGGGTACAACAGGATCTTGGCGAC</td>
<td>Reverse primer used for PCR genotyping of ∆prpDC allele</td>
<td>1195bp amplicon from ∆prpDC allele using PrpDC-F and PrpDC-R2</td>
</tr>
<tr>
<td>Icl1-F</td>
<td>GGGGGACAACGCTCACATATGTGGTT</td>
<td>Forward and reverse primer pair used to generate the DNA probe used for genotypic confirmation of the ∆icll mutant by Southern blot analysis</td>
<td>787bp amplicon generated from wild type icll allele using Icl1-F and Icl1-R</td>
</tr>
<tr>
<td>Icl1-R</td>
<td>GGGGGAGCCAGTTCTCCACCGAAGTA</td>
<td>Forward and reverse primer pair used to generate the DNA probe used for genotypic confirmation of the ∆icll mutant by Southern blot analysis</td>
<td></td>
</tr>
</tbody>
</table>
Forward and reverse primer pair used to generate a full length copy of \( \text{Rv1998c} \) for expression study. 862bp PCR product restricted with \( \text{SphI} \) and \( \text{BamHI} \) and cloned into \( \text{pSE100} \). Primers used to sequence PCR product.

Primer pair used for PCR to confirm the presence of episomal \( \text{pSE100::Rv1998c} \) in \( \Delta \text{icl1} \) :: \( \text{hyg} \) :: \( \text{pMC1m::Pmyc\_tetO::Rv1998c} \) in \( \Delta \text{icl1} \) :: \( \text{hyg} \) :: \( \text{pMC1m::Pmyc\_tetO::Rv1998c} \) in \( \Delta \text{icl1} \) :: \( \text{hyg} \) :: \( \text{pMC1m::Pmyc\_tetO::Rv1998c} \)

862bp amplicon generated from wildtype \( \text{Rv1998c} \) allele using \( \text{pSE100} \) and \( \text{Rv1998c}\_F \) and \( \text{Rv1998c}\_R \) and \( \text{pSE100Rv1998c-F} \) and \( \text{pSE100Rv1998c-R} \)

862bp amplicon

388bp amplicon

500bp amplicon

799bp amplicon

799bp amplicon

857bp amplicon

1130bp amplicon

500bp amplicon

799bp amplicon

799bp amplicon
### RT primers

<table>
<thead>
<tr>
<th></th>
<th>Primer Sequence</th>
<th>Amplicon Length</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998c RT2-F</td>
<td>GCTATCGGCACAACCAGTTT</td>
<td>189bp</td>
<td>189bp amplicon to detect AHTc induced expression of Rv1998c</td>
</tr>
<tr>
<td>1998c RT2-R</td>
<td>CGACAGTTGTCGCAGTGTAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutB RT-F</td>
<td>TATCGTTCTTCGGGGGATC</td>
<td>187bp</td>
<td>187bp amplicon</td>
</tr>
<tr>
<td>mutB RT-R</td>
<td>GCCACGTTGTTGACACATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PrpD RT(AS)</td>
<td>ATCAGCTGGTAGATGGTCTC</td>
<td>155bp</td>
<td></td>
</tr>
<tr>
<td>prpD RT (S)</td>
<td>GGTCTGGGACCCCACTATGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ic11 RT-R</td>
<td>ATACGGCGCTCATCTGGTCTC</td>
<td>195bp</td>
<td></td>
</tr>
<tr>
<td>ic11 RT-F</td>
<td>ATGCTGGCCCTACAACGCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sigA-F1</td>
<td>TGGACGACGCTGCTGGGACAC</td>
<td>194bp</td>
<td></td>
</tr>
<tr>
<td>sigA-R1</td>
<td>CGGCAGGACACGTGAGCGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Dawes et al., 2003)

### Sequencing primers for Rv1129c and prpDC region

<table>
<thead>
<tr>
<th></th>
<th>Primer Sequence</th>
<th>Amplicon Length</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv1129c-F</td>
<td>AGCCACCGGAAGAGCATAC</td>
<td>PCR and sequencing primer</td>
<td>1679bp PCR product using Rv1129cF and Rv1131R from two isolated Δicl1 suppressor mutants and sequenced</td>
</tr>
<tr>
<td>Rv1131-R</td>
<td>TCATCGATATTTGTCAGCGA</td>
<td>PCR and sequencing primer</td>
<td></td>
</tr>
<tr>
<td>Rv1130-F</td>
<td>TGGACGCTGGTAGATGGTCTC</td>
<td>PCR and sequencing primer</td>
<td>1276bp PCR product using Rv1130 F and Rv1132 R from two isolated Δicl1 suppressor mutants and sequenced</td>
</tr>
<tr>
<td>Rv1132-R</td>
<td>ATCCGACGCTGAGTCGCGA</td>
<td>PCR and sequencing primer</td>
<td></td>
</tr>
<tr>
<td>Rv1128c-F</td>
<td>TGGCTGAAACCACACGTAATAT</td>
<td>PCR and sequencing primer</td>
<td>1684bp PCR product generated using Rv1128c and PrpDC-R from two isolated Δicl1 suppressor mutants and sequenced</td>
</tr>
<tr>
<td>Rv1130a -R</td>
<td>ATCCGCTGGTAGATGGTCTT</td>
<td>sequencing primer</td>
<td>Used to sequence Rv1130 amplified from two isolated Δicl1 suppressor mutants</td>
</tr>
<tr>
<td>Rv1130b -F</td>
<td>TAACCGCTATGAGATCCACAT</td>
<td>sequencing primer</td>
<td>Used to sequence Rv1130 amplified from two isolated Δicl1 suppressor mutants</td>
</tr>
<tr>
<td>Rv1130c -F</td>
<td>ATGTTAGTGACGGAAACCGGAT</td>
<td>sequencing primer</td>
<td>Used to sequence Rv1130 amplified from Δicl1 1 and 2 suppressor mutants</td>
</tr>
<tr>
<td>Rv1131a -R</td>
<td>TGGCTAGCGCTGAGTCGGA</td>
<td>sequencing primer</td>
<td>Used to sequence Rv1131 amplifier</td>
</tr>
</tbody>
</table>

160
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv1131b-F</td>
<td>TTATGCGCAGAACTTCTGCACA</td>
<td></td>
<td>sequencing primer Used to sequence Rv1131 amplified from two isolated Δicl1 suppressor mutants</td>
</tr>
<tr>
<td>Rv1131c-F</td>
<td>AAGCTGCCGGACAACTGCCA</td>
<td></td>
<td>sequencing primer Used to sequence Rv1131 amplified from two isolated Δicl1 suppressor mutants</td>
</tr>
<tr>
<td>Rv1131d-R</td>
<td>AGGAAGGCGACCTGCTCGAAACT</td>
<td></td>
<td>sequencing primer Used to sequence Rv1131 amplified from two isolated Δicl1 suppressor mutants</td>
</tr>
</tbody>
</table>

a. GC-clamp sequences (non-H37Rv) are italicised. Restriction sites used for subsequent cloning of PCR fragments are underlined, and the relevant enzyme detailed under application.
All things are difficult before they are easy.
Thomas Fuller, M.D.