Molecular mechanisms of transport and metabolism of vitamin B_{12} in mycobacteria

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"Then which of the favours of your Lord will you deny?"

Quran
Chapter 55, Verse 13
Surah Ar-Rahman (The Beneficent)

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.

9 Oct 2012

For my parents

Azeeza and Johnny van Silk

And my sister

Ayesha

You fill my life with light and love

Abstract

Mycobacterium tuberculosis (MTB) encodes three enzymes that are dependent on vitamin B_{12}–derived cofactors for activity, including a B_{12}-dependent methionine synthase (MetH). Previously, work in the Molecular Mycobacteriology Research Unit (MMRU) demonstrated vitamin B_{12} auxotrophy in a mutant strain disrupted in the alternative, B_{12}-independent methionine synthase, MetE. This observation established the ability of MTB to transport corrinoids despite the absence of an identifiable B_{12}-specific transporter. In addition, it suggested that MTB does not synthesize vitamin B_{12} *in vitro.* Notably, bioinformatic analyses identified PPE2 as the only B_{12}-related transport candidate in MTB, though as a putative B_{12}-regulated cobalt transporter. *PPE2* is unusual in possessing directly upstream of its predicted start codon one of only two B_{12}-dependent riboswitches in the MTB genome, and it lies in a putative operon with B_{12} biosynthetic genes, *cobU* and *cobQ1*. In this study, the possibility that *PPE2* functions in the transport of vitamin B_{12} or cobalt was investigated. Transcriptional and phenotypic data suggested that PPE2 was not involved in B_{12} transport. Instead, it was shown that cobalt can supplement the growth of an MTB *metE* mutant in liquid medium, strongly supporting the ability of MTB to synthesize B_{12} *de novo*. Moreover, the ability to utilise exogenous cobalt was dependent on functional PPE2, thereby establishing a role for a PPE-family member in cobalt assimilation in MTB.

Vitamin B_{12} comprises a central corrin ring co-ordinated to 5,6-dimethylbenzimidazole (DMB) as α-axial ligand. Substituting DMB with adenine yields the alternate form, pseudo-B_{12}. The ability of mycobacteria to utilize pseudo-B_{12} precursors (cobinamide and adenine) to support full function of B_{12}-dependent metabolic pathways was evaluated. Although the pseudo-B_{12} precursors appeared to complement chemically the mycobacterial B_{12} auxotrophs, growth of the mutants on cobinamide alone

complicated this interpretation. To address this limitation, DMB synthesis was targeted by disrupting the MTB *bluB* homologue, *Rv0306*. Neither site-directed mutagenesis of key Rv0306 residues, nor full-gene deletion was sufficient to eliminate growth on cobinamide. Instead, this observation highlights the need to establish biochemically the nature of the active B_{12} form synthesized and utilized by MTB under different conditions.

In combination, the results presented here support the inferred flexibility of vitamin B_{12} biosynthesis in MTB, and reinforce the potential role of B_{12}-dependent metabolism in mycobacterial pathogenesis.

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Table of Contents

List of Figures

List of Tables

1. Introduction

1.1 Tuberculosis

Mycobacterium tuberculosis (MTB) has long been the scourge of humanity as it causes one of the most devastating diseases, tuberculosis (TB). This air-borne bacterial pathogen claimed a staggering 1.7 million lives in 2009 alone (WHO, 2009). Although statistics indicate that the global incidence and deaths due to TB are decreasing, this is sadly not true for developing countries which continue to shoulder the weight of this burden, accounting for more than 90% of all new cases as well as TB-related deaths (WHO, 2009). In resource-poor settings, poor socio-economic conditions are exacerbated by concurrent infection with human immunodeficiency virus (HIV); in fact, countries with the highest prevalence of HIV are the worst affected by the TB burden (WHO, 2009). Another primary source of concern is the rapid emergence globally of, and alarming increase in, drug resistant MTB strains which further threaten the control and elimination of TB. Despite on-going preventative measures including the administration of the BCG vaccine, the availability of effective chemotherapeutic regimens as well as worldwide support programs, TB continues to be a massive global health crisis.

1.2 Prevention and chemotherapy

1.2.1 The inadequacies of BCG

The current TB pandemic is testament to the critical limitations of the only vaccine used to prevent TB, the live-attenuated bacille Calmette-Guérin (BCG). This vaccine was derived by *in vitro* passage of a *Mycobacterium bovis* isolate over a number of years. Despite the administration of BCG to

more than 3 billion people worldwide (Brosch *et al.*, 2000), its efficacy remains inconsistent: the protective effect in various clinical trials ranged from 80% to nil (Fine, 1989), with several theories offered to explain this variability (Colditz *et al.*, 1994; Fine, 1995; Brewer, 2000; Behr, 2001; Brosch *et al.*, 2007). Whilst there is consensus that BCG is highly effective in protecting against severe childhood forms of TB (Colditz *et al.*, 1995), it offers no protection against the most common form of the disease - that is, adult pulmonary TB which represents the main source of new infections and is the greatest contributor to mortality (WHO, 2009). In addition, there is a higher risk of vaccine-related complications such as disseminated BCG disease in HIV-infected children (Hesseling *et al.*, 2007; Hesseling *et al.*, 2009). This prompted the World Health Organization (WHO) Global Advisory Committee on Vaccine Safety to contraindicate BCG administration in HIV-infected and HIV-exposed children (WHO, 2007). However, not vaccinating an HIV-exposed infant who remains uninfected increases the risk of disseminated TB; therefore, BCG continues to be administered in settings where TB and HIV are highly endemic (WHO, 2007; Hesseling *et al.*, 2009).

These concerns highlight the urgent need for safer and more effective vaccines which will aid in controlling the disease. Amongst the challenges facing new vaccine development is the need to design a vaccine that will not only elicit a strong host immune response to the initial infection (pre-exposure vaccine), but will prevent progression to disease in those previously exposed (post-exposure vaccine) (Brennan, 2005). Perhaps even more challenging is the development of a vaccine that is safe for use in HIV-infected populations. It is encouraging, therefore, that the last few years have seen substantial progress in this area: there are several candidate TB vaccines in the pipeline, in clinical trials or on their way to preclinical development (Grode *et al.*, 2005; Tullius *et al.*, 2008; STOPTB,

2009; Aagaard *et al.*, 2011). Ideally, new vaccines should complement drug regimens as this will significantly aid in the fight against this disease.

1.2.2 Chemotherapy and drug resistance

TB is curable; however, chemotherapy faces its own set of challenges that prevent the universal control of this disease. As part of an internationally recommended strategy to accelerate the control and eradication of TB worldwide, the WHO implemented directly observed therapy, short course (DOTS) in the early 1990's. The foundation of this control strategy is a short-course treatment comprising a daily dose of a combination of drugs over a period of six months, with administration of each dose supervised by a health care worker. The short-course treatment regimen comprises an intense phase of isoniazid, rifampicin, pyrazinamide and ethambutol for two months, followed by a continuation phase of isoniazid and rifampicin for an additional four months (WHO, 2010). When all five elements (strong government support, effective case detection, standardized treatment with supervision, continued drug supply, and surveillance) are implemented in their entirety, chemotherapy under DOTS has been shown to achieve high cure rates for drug-sensitive TB (Murray, 1996). However, this requires substantial resources and a strong health infrastructure (Espinal and Dye, 2005). Moreover, in the context of high TB prevalance, the DOTS programme is probably inappropriate (Wood *et al.*, 2011).

A combination of inadequate drug regimens as well as the lengthy duration of therapy, results in inconsistent or even incomplete treatment, which in turn promotes the selection and spread of drug resistant strains that are difficult to treat. Multidrug resistant strains (MDR) are resistant to two of the most powerful first-line drugs - isonaizid and rifampicin - and so necessitate treatment with second-line drugs as part of the so-called

"DOTS plus" programme (WHO, 2010). An expansion of DOTS, this programme has been tailored to the management and treatment of MDR-TB, specifically taking into account resource-poor countries with high HIV co-incidence. Treatment of MDR-TB is much more protracted, more expensive, more toxic and is considerably more complicated as some drugs are administered by injection; in combination, these factors result in lower cure rates and increased spread of the disease (Espinal and Dye, 2005; Marahatta, 2010).

Given these complexities in treating MDR, the emergence of extensively drug resistant (XDR) strains of TB, which are resistant to isonaizid, rifampicin, any fluoroquinolone, and at least one of the three injectable second-line drugs (amikacin, kanamycin and capreomycin) seems almost inevitable (CDC, 2006). That said, the rapid lethality observed in an outbreak of XDR-TB among HIV-positive individuals in the town of Tugela Ferry located in the central KwaZulu-Natal province, South Africa, was alarming (Gandhi *et al.*, 2006). The startling report by Gandhi *et al.* (2006) revealed that, of the 475 confirmed TB cases, 53 individuals (of whom 44 were HIV positive) had XDR-TB. Fifty-two of these died within 16 days of diagnosis. Notably, exogenous re-infection with MDR- or XDR-TB strains was implicated in the development of this outbreak (Andrews *et al.*, 2008), suggesting poor infection control practices, lack of proper diagnostic testing, and ineffective treatment of susceptible and MDR-TB (Jassal and Bishai, 2009). Sadly, XDR-TB is very difficult to treat, requiring extended therapy (18-24 months) with poor outcomes and significantly increased costs (Chan *et al.*, 2008). Even more distressing are the recent reports describing the emergence of totally drug resistant (TDR) strains, and the possible catastrophic implications for treatment and control of this form of TB (Velayati *et al.*, 2009). Another area of concern is the treatment of TB in patients receiving anti-retroviral therapy due to unfavourable drug interactions, overlapping toxicities, high pill burden and

the potential for the development of immune reconstitution inflammatory syndrome (IRIS) (McIlleron *et al.*, 2007).

In combination, these elements are driving the need for new TB drugs. This has mobilized organizations like the WHO and the Global Alliance for TB Drug Development to dedicate enormous efforts and funding towards the control and eradication of TB. The WHO implemented the "Global Plan to STOP TB 2006-2015" which is a program that aims to halve the global TB prevalence by 2015 relative to 1990, and to eliminate TB as a public health problem by 2050 (STOPTB, 2006). Additionally, the TB Alliance aims to reduce the duration of chemotherapy from 6-9 months to 2 months or less, as this will improve patient adherence to therapy and thereby prevent further drug resistance (TBAlliance, 2005). Together, these organizations have been instrumental in stimulating and accelerating the discovery and development of new anti-tuberculosis drugs. Although no new drugs are expected at the dispensary soon, there are some promising candidates in the pipeline which include two nitroimidazole compounds (PA-824 and OPC-67683) and a diarylquinoline compound (R207910, also called TMC207) (Stover *et al.*, 2000; Andries *et al.*, 2005; Matsumoto *et al.*, 2006; Koul *et al.*, 2011). To ensure that this pipeline is populated, basic research is required that is aimed at further understanding TB biology at different stages of infection, as well as this pathogen's extraordinary ability to subvert host immune responses.

1.3 Survival of MTB within the host

1.3.1 Intramacrophage survival

MTB's success can be attributed to its ability to blunt the host immune response, and then establish a niche within the host for extended periods despite the hostile environments and continual immune surveillance (Nguyen and Pieters, 2005; Ahmad, 2011). By far the most common route of infection of MTB is through inhalation of aerosolized bacilli, which are subsequently ingested and enclosed within a phagosome within alveolar macrophages in the lower airways (Clark-Curtiss and Haydel, 2003). Entry of MTB into macrophages is facilitated through a variety of receptors including complement receptors (CR3, CR1, CR4), mannose receptors, Fc receptors and toll-like receptor (TLR) (Astarie-Dequeker *et al.*, 1999; Hingley-Wilson *et al.*, 2000; van Crevel *et al.*, 2002; Pieters, 2008). It has been suggested that internalization of MTB through certain receptors is beneficial and may assist in circumventing immediate destruction once inside the macrophage. For example, ingestion through the Fc receptor triggers an inflammatory response, as opposed to entry via the CR3 receptor which appears to avert macrophage activation (Caron and Hall, 1998; Pieters, 2008).

1.3.2 Immune evasion and granuloma formation

Ingestion by activated macrophages results in the gradual acidification of the phagosome which fuses with lysosomes to form a phagolysosome (phagosome maturation) loaded with proteolytic enzymes. This is accompanied by a simultaneous increase in the generation of reactive oxygen and nitrogen intermediates which target multiple cellular structures and biochemical components to bring about the degradation

and death of the bacilli (Smith, 2003; Hestvik *et al.*, 2005; Nguyen and Pieters, 2005). However, in the case of inactivated macrophages, MTB has evolved strategies that avoid destruction as well as allow survival and growth within the phagosome. These include prevention of phagosome-lysosome fusion through a series of complex events that result in the retardation of phagosome maturation (Armstrong and Hart, 1971; Sturgill-Koszycki *et al.*, 1994; Via *et al.*, 1997; Malik *et al.*, 2000; Vergne *et al.*, 2005; Jayachandran *et al.*, 2007), the use of specific receptors that do not trigger an oxidative burst upon entry into the phagosome (Wright and Silverstein, 1983; Schlesinger *et al.*, 1990), resistance to reactive nitrogen intermediates (Miller *et al.*, 2004), down-regulation of host immune modulators (Ting *et al.*, 1999; Noss *et al.*, 2000; Hickman *et al.*, 2002; Nau *et al.*, 2002), and blocking of apoptosis (Sly *et al.*, 2003).

As a result, MTB avoids elimination and continues proliferating intracellularly until phagosomal lysis occurs which releases viable bacilli into the surrounding lung tissue, where they are subsequently ingested by other macrophages (Clark-Curtiss and Haydel, 2003; Grosset, 2003). These immune cells similarly fail to control growth of MTB and are destroyed. Inflammatory cytokines and chemokines released by lysed macrophages stimulate the recruitment of additional monocyte-derived macrophages and dendritic cells to the site of infection where they readily ingest, but do not destroy, mycobacteria (van Crevel *et al.*, 2002). During this time, dendritic cells with engulfed bacilli mature and migrate to lymph nodes where they prime T cells ($CD4^+$ and $CD8^+$) against mycobacterial antigens (Tufariello *et al.*, 2003). Two to eight weeks post infection, a cell-mediated immune response arises whereby primed T-cells migrate back to the site of infection in the lungs and activate defence mechanisms of macrophages containing MTB to destroy the invading bacilli or at least contain their growth (Saunders *et al.*, 1999; Grosset, 2003; Ahmad, 2011). Moreover, T-cells strengthen antibacterial responses by recruiting and

activating additional immune cells, resulting in an influx of monocytes to the site of infection. The ensuing battle between the host immune system and MTB leads to the cessation of bacterial growth, and infiltration of immune cells that aggregate around the infected tissue to form a solid structure or a granuloma (Bloom and Murray, 1992; Clark-Curtiss and Haydel, 2003), that is sealed off from surrounding tissues by a fibrotic capsule (Stewart *et al.*, 2003). Within the barricade-like structure of the granuloma, bacterial dissemination is prevented, and interactions between T-cells, macrophages and cytokines are facilitated which result in control of the infection in most immunocompetent individuals (Saunders *et al.*, 1999; Tufariello *et al.*, 2003).

1.3.3 Latent TB and metabolic readjustments

In about 10% of infected individuals, bacilli continue to proliferate – presumably due to ineffective T-cell responses – which results in the development of TB within 1-2 years (Caruso *et al.*, 1999; Dye *et al.*, 1999). The remaining 90% successfully control the infection; however, a residual proportion of bacilli persist for extended periods within the granuloma (Ducati *et al.*, 2006). The only clinical evidence of a latent TB infection (LTBI) is a delayed-type hypersensitivity response against mycobacterial antigens, demonstrated by the tuberculin skin test (Glickman and Jacobs Jr, 2001). Latently infected individuals are asymptomatic; however, several factors including HIV, cancer, diabetes, drug use, and malnutrition are known to favour reactivation and clinical manifestation of MTB (Parrish *et al.*, 1998; Cosma *et al.*, 2004).

Although poorly understood, during LTBI bacilli within granulomas are thought to be characterized by decreased metabolic activity and limited or no replication (Hu *et al.*, 2000; Muñoz-Elías and McKinney, 2005; Gill *et*

al., 2009). The environment within the granuloma is hostile with reduced availability of oxygen, acidic pH, the presence of toxic fatty acids, nitric oxide stress, and scarce supply of nutrients (Smith, 2003), all of which are thought to drive MTB into a non- or slowly replicating, drug-tolerant state. Several *in vitro* and *in vivo* models including the Wayne model (Wayne and Hayes, 1996; Wayne and Sohaskey, 2001), nutrient starvation model (Betts *et al.*, 2002), Cornell mouse model (McCune *et al.*, 1956), and low-dose murine model (Orme, 1988) have been developed to recreate aspects of this state, as latency in the human host can take years to manifest and is not experimentally tractable. Although individually these models do not fully emulate the scenario between the host and bacterium during infection, they have proved very useful in investigating the metabolism and physiology of MTB under multiple conditions and stresses thought to prevail during chronic, persistent infection.

Together with phenotypic, transcriptomic and proteomic analyses utilizing model systems, studies have identified various genes induced under conditions of non-replicating persistence. Overall, these observations indicate that under nutrient starvation, low oxygen, and nitrosative stress, MTB utilizes many mechanisms that allow it to undergo significant metabolic reprogramming to survive during chronic infection. Some of these include the induction of stress-related pathways including the dormancy regulon which is comprised of approximately 50 genes under the control of the *dosR/S/T* two-component regulatory system and is induced during hypoxia and upon exposure to low-dose nitric oxide or to carbon monoxide (Schnappinger *et al.*, 2003; Voskuil *et al.*, 2003; Kendall *et al.*, 2004; Roberts *et al.*, 2004; Voskuil *et al.*, 2004a). The α-crystallin like protein *hspX* which is induced in response to stationary phase, low oxygen and nitrosative stress (Yuan *et al.*, 1996); (Cunningham and Spreadbury, 1998; DesJardin *et al.*, 2001) and the Rel_{Mtb}-regulated stringent response to nutrient starvation (Primm *et al.*, 2000; Betts *et al.*,

2002). Additionally, mycobacterial factors involved in cell wall modifications (*pcaA*) (Glickman *et al.*, 2000), anaerobic energy metabolism (*narX*) (Hutter and Dick, 1999; Sherman *et al.*, 2001) and lipid metabolism (*icl*) (Wayne and Lin, 1982) have also been identified as playing a role in persistence.

1.4 Carbon metabolism in MTB

Seminal experiments by Segal and Bloch (1956) suggested the importance of carbon metabolism to mycobacterial physiology; however, the extent of the role of alternate carbon sources in MTB has only been realized and fully appreciated recently. Like many bacterial species, MTB can metabolize a variety of carbon sources to generate energy during growth in synthetic media (Wheeler and Ratledge, 1994). This is consistent with the presence of enzymes required for glycolysis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, the glyoxlate cycle and the methylcitrate pathway in MTB (Cole *et al.*, 1998). One of the main challenges faced by MTB during infection is the need to acquire nutrients from host cells. Even though carbohydrate and lipid metabolism genes have been shown to be transcribed during infection (Talaat *et al.*, 2007), the types of nutrients available to MTB within the host remain unclear. However, emerging evidence has implicated lipids as the dominant carbon source.

1.4.1 Lipids as the dominant carbon source

Early studies by Segal and Bloch (1956) demonstrated that bacteria isolated from mouse lung responded to substrates containing fatty acids, whereas broth-grown bacilli responded to a variety of carbon sources. This was the first indication that MTB obtained its energy from the metabolism of fatty acids as opposed to carbohydrates during infection. Subsequently, Wayne *et al.* (Wayne, 1977; Wayne and Hayes, 1996) developed the *in vitro* model of latency based on the idea that MTB adapts to the oxygen-deficient environment within the granuloma. During the metabolic shift that occurs as a result of gradual oxygen depletion, enzymes involved in the glyoxlate shunt are up-regulated (Wayne and Lin, 1982). The glyoxylate shunt is an anaplerotic pathway that consists of two enzymes, isocitrate lyases (ICL) and a malate synthase (GlcB), which facilitate the conversion of fatty acids into carbohydrates by bypassing the two oxidative steps of the TCA cycle (Wayne, 1994). Furthermore, a complex repertoire of more than 250 genes involved in lipid metabolism is present in the MTB genome (Cole *et al.*, 1998). Genes involved in lipid metabolism, together with those encoding glyoxylate cycle enzymes, are up-regulated during infection of macrophages (Schnappinger *et al.*, 2003) and mice (Timm *et al.*, 2003; Dubnau *et al.*, 2005).

Fatty acids can be catabolized via the glyoxylate shunt and the β-oxidation cycle; however, the latter is the dominant pathway utilized in bacteria (McKinney *et al.*, 2000; Muñoz Elías and McKinney, 2006; Marrero *et al.*, 2010). Catabolism of fatty acids and lipids via β-oxidation generates acetyl-CoA that is shunted into the glyoxylate cycle to prevent carbon loss via the TCA cycle; this produces succinate which can be converted into glucose (Manabe and Bishai, 2000; Bentrup and Russell, 2001; Muñoz Elías and McKinney, 2006). Carbon flux into the glyoxylate cycle is mediated by ICL, which catalyzes the conversion of isocitrate to succinate

and glyoxylate and, in MTB Erdman, is encoded by two genes, *icl1* and *icl2*. In contrast, H37Rv has a single ICL, encoded by *icl1* (Muñoz-Elías and McKinney, 2005). When both isoforms, ICL1 and ICL2 were absent, the Erdman strain of MTB was able to grow on carbohydrates but incapable of growth on fatty acids or in macrophages, and was rapidly eliminated from the lungs of infected mice (Muñoz-Elías and McKinney, 2005). These data suggest that the glyoxlate shunt is essential for survival during murine chronic infection. In addition, oxaloacetate (OAA) from the glyxolate cycle can be fed into gluconeogensis, where it is converted to phosphoenolpyruvate (PEP) by the *pckA*-encoded phosphoenolpyruvate carboxykinase (PEPCK). A *pckA* deletion mutant of MTB was impaired for growth on fatty acids but not glucose *in vitro* (Marrero *et al.*, 2010), indicating that this enzyme does not function in the reverse direction in MTB; that is, the conversion of PEP to OAA (Marrero *et al.*, 2010). PEPCK was also shown to be required for growth and survival in macrophages and mice (Marrero *et al.*, 2010), and was induced by fatty acids *in vitro* (Schnappinger *et al.*, 2003; Dubnau *et al.*, 2005), and during growth of MTB in mice (Timm *et al.*, 2003). Together these data strenthen the idea that MTB subsists on fatty acids *in vivo*.

1.4.2 Sources of lipids during infection

The ability of MTB to shift its metabolism to utilize lipids as a carbon source appears to be a key strategy for persistence and survival during the chronic phase of infection. The types of lipids available to MTB within the varied microenvironments in the host remain undefined; however, possible sources of lipids include lung surfactant internalized by alveolar macrophages that is rich in long chain fatty acids (Muñoz Elías and McKinney, 2006), hydrolysis of lipids from the phagosomal membrane (Kondo *et al.*, 1985; Muñoz Elías *et al.*, 2006), macrophage triacylglycerol

(TAG) stores (Mason *et al.*, 1972), or accumulated internal MTB TAG stores (Daniel *et al.*, 2004).

Emerging evidence suggests that cholesterol, a major sterol of the plasma membrane, is abundantly available as an alternate carbon source within the host. Studies have demonstrated that accumulation of cholesterol is necessary at the site mycobacterial entry (Gatfield and Pieters, 2000) and depletion inhibits cellular uptake into phagocytic cells (Nguyen and Pieters, 2005), as well as stimulates phagosomal maturatation leading to decreased survival of bacilli (De Chastellier and Thilo, 2006). Pathogenic mycobacteria have been shown to induce the formation of foamy macrophages filled with lipid bodies which are known to accumulate in human and mouse granulomas (Cardona *et al.*, 2000; Peyron *et al.*, 2008; Russell *et al.*, 2009). It was also demonstrated by electron microscopy that bacilli are positioned in proximity to lipid bodies within foamy macrophages and these are hypothesized to serve as a nutrient-rich reservoir during persistence (Peyron *et al.*, 2008). Furthermore, biochemical anaylsis of the lipid species within the caseum revealed an abundance of cholesterol ester, cholesterol and triglycerol (Kim *et al.*, 2010).

1.4.2.1 Evidence for cholesterol utilization

Although several other lines of evidence have implicated cholesterol as a carbon source for MTB, an important study by Pandey and Sassetti (2008) provided the first genetic evidence that this pathogen mobilizes and catabolizes cholesterol from the host. With the aid of metabolic labelling studies, these authors reported that MTB can catabolize different portions of the cholesterol molecule for energy generation and the synthesis of the virulence factor, phthiocerol dimycocerosate (PDIM) (Pandey and Sassetti,

2008). Deletion of the *mec4*-encoded cholesterol transporter (Mohn *et al.*, 2008) resulted in poor growth in media containing cholesterol as a sole carbon source. More interestingly, a MTB mutant lacking *mcec4* displayed impaired survival during the chronic phase of infection in mice, and in interferon-gamma activated macrophages (Pandey and Sassetti, 2008). Similar to the phenotype observed for the Δ*icl1* mutant of MTB Erdman (McKinney *et al.*, 2000), loss of *mce4* function had no effect on growth in non-activated macrophages or during the acute phase of murine infection. These data suggest that cholesterol is an important nutrient source during chronic infection when interferon-gamma macrophages are activated. However, it remains unclear what constitutes the major nutrient source for MTB during earlier stages of infection.

Subsequently, a study by Chang and collegues (2009) found that deletion of the intracellular growth operon (*igr*) inhibited growth of MTB in cholesterol-containing media, whereas growth on short- and long- chain fatty acids was unaffected. Elimination of the Mce4 transporter in the Δ*igr* mutant partially reversed this cholesterol-dependent phenotype (Chang *et al.*, 2009). Moreover, the Δ*igr* mutant displayed attenuation in mice during the early phase of infection, possibly due to accumulation of toxic intermediates produced by cholesterol catatabolism. Conversely, disruption of the cholesterol import system (*mce4*) in the Δ*igr* mutant resulted in attenuation during late phase murine infection, reinforcing the importance of cholesterol during infection. Recently, the *igr* operon was shown to be necessary for the degradation of the 2'-propanoate side chain of cholesterol metabolites by MTB (Thomas *et al.*, 2011). These results are consistent with the idea that, in mice (Miner *et al.*, 2009) and guinea pigs (Yang *et al.*, 2011), cholesterol is avaliable throughout infection and only becomes limiting during the chronic phase – after the onset of adaptive immunity has altered the environment. Importantly, infection of guinea pigs with an MTB strain deficient in an iron-dependent extradiol

dioxygenase (*hsaC*), a key enzyme in cholesterol catabolism (Van der Geize *et al.*, 2007), resulted in slower dissemination, decreased persistence, and reduced pathology in the lungs (Yam *et al.*, 2009). Together, these studies confirm that cholesterol utilization by MTB is important in chronic disease as well as earlier in infection and suggest that this sterol is likely to contribute to the pathogen's ability to disseminate in the host. This conclusion is supported by the recent identification of an expanded set of genes which are predicted to be essential for cholesterol-dependent growth of MTB (Griffin *et al.*, 2011).

1.4.3 Toxicity associated with lipid metabolism

Beta-oxidation of even-chain fatty acids yields acetyl-CoA, whilst β-oxidation of cholesterol, odd- and branched- chain fatty acids produces propionyl-CoA in addition to acetyl-CoA (Muñoz Elías *et al.*, 2006). Propionyl-CoA can be oxidized to pyruvate via the methylcitrate cycle, whose specific enzymes, methylcitrate synthase and methycitrate dehydrogenase, are encoded by *prpC* and *prpD*, respectively. Muñoz-Elías and McKinney (2006) observed that a MTB *prpDC* mutant was unable to grow on odd-chain fatty acids such as propionate and valerate, but was able to grow on even chain fatty acids. Together with their previous observation that a Δ*icl1* Δ*icl2* double mutant of MTB mutant was unable to grow on either odd- or even- chain fatty acids (Muñoz-Elías and McKinney, 2005), this led the authors to conclude that ICL1 could function as methylisocitrate lyase. Together these results also demonstrated the essentiality of the methylcitrate cycle in MTB in detoxifying propionate, and reinforced the notion that, although propionate is a high-energy metabolite, its accumulation is toxic to the bacterial cell. For this reason, it is likely that propionate metabolism fulfils an essential function in

preventing the toxic build-up of propionyl-CoA within the cell. It was surprising, therefore, that a *prpDC* mutant of MTB Erdman showed no defect in growth or persistence in a mouse model of infection (Muñoz Elías and McKinney, 2006), in contrast to a Δ*icl1* mutant, which showed a persistence defect (McKinney *et al.*, 2000) and a Δ*icl1* Δ*icl2* double mutant, which showed a dramatic phenotype, being unable even to establish an infection (Muñoz-Elías and McKinney, 2005). Besides confirming the critical role of ICL in disease pathogenesis, these findings suggested that propionyl-CoA detoxification *in vivo* could occur via a route(s) other than through the methylcitrate cycle (Muñoz-Elías and McKinney, 2005). That interpretation has gained greater credence with the recent demonstration that survival of an MTB *prpDC* mutant in a macrophage model of infection is enhanced by the addition of vitamin B_{12} to the growth medium (Griffin *et al.*, 2012). In particular, this result suggests that the B_{12}-dependent methylmalonyl pathway might function to alleviate propionate stress *in vivo*, which in turn implies that MTB might be able to synthesize - or access - vitamin B_{12} during host infection.

The first step in the methylmalonyl pathway involves the conversion of propionyl-CoA to (*S*)-methylmalonyl-CoA via the propionyl-CoA carboxylase (PCC) enzyme. This provides intermediates (methylmalonyl-CoA) for the biosynthesis of methyl-branched lipids such as PDIM and sulfolipid (SL)-1 (Yang *et al.*, 2009). These surface exposed lipids form an integral part of MTB's cell wall as they provide protection against host-induced damage, as well as modulate the immune response (Reed *et al.*, 2004; Rousseau *et al.*, 2004), and so have been implicated in virulence. Recently, it was demonstrated that MTB grown on propionate or odd-chain fatty acids displayed increased mass and abundance of PDIM and SL-1; however, no increase was observed when grown on short- and even- chain fatty acids (Jain *et al.*, 2007). In fact, growth on propionate or odd- chain fatty acids resulted in the extension of PDIM mycocerosic acids

by 3-carbons, which in turn suggests the direct incorporation of the propionyl-CoA intermediate methylmalonyl-CoA. Interestingly, PDIM isolated from wild-type MTB in mouse lungs also displayed increased mass and lengthened mycocerosic acids. This result might indicate comparable propionyl-CoA build up as a result of β-oxidation of host lipids that increased the flux of methylmalonyl-CoA through lipid biosynthetic pathways which in turn increased virulence lipid biosynthesis.

In another recent study, it was reported that MTB responds to redox fluctuations by selectively incorporating C3 compounds such as propionate or propionyl-CoA into cell wall lipids like polyacyltrehaloses (PAT), PDIM, SL-1 and the storage lipid TAG under the control of the DosR/S/T dormancy regulon (Singh *et al.*, 2009). This C3 compound incorporation was observed in murine macrophage infections and was shown to be controlled by the transcriptional regulator WhiB3, which operates via a thiol-disulphide redox switch. Supporting the role of WhiB3 as a regulator of mycobacterial lipids, microarray data indicate up-regulation of WhiB3 together with genes responsible for the production of SL-1, PAT, DAT and TAG in macrophages (Rohde *et al.*, 2007). A MTB WhiB3 deletion mutant was able to grow even on toxic concentrations of propionate; in addition, this mutant was associated with increased levels of PDIM during growth in liquid (7H9/OADC) culture and within macrophages. Therefore, the possibility remains that MTB might differentially incorporate C3 compounds into cell wall lipids as a means of detoxification.

In addition to detoxifying excess intracellular propionate into cell wall lipids via the first steps of the methylmalonyl pathway, the last step of this pathway can be utilized to catabolise propionyl-CoA for energy generation. This step entails the isomerization of (*R*)-methylmalonyl-CoA to (S)-methylmalonyl-CoA, which is converted to succinyl-CoA (Savvi *et al.*, 2008) in a reaction catalyzed by the *mutAB*-encoded methylmalonyl-CoA

mutase, a B_{12}-dependent enzyme (Savvi *et al.*, 2008). The succinyl-CoA can be fed into the TCA or glyoxylate cycles. Previously, Muñoz-Elías and McKinney (2005) demonstrated that treatment of MTB with the ICL inhibitor, 3-nitropropionate (3-NP), resulted in growth inhibition in media containing fatty acids, and that this phenocopied the Δ*icl1* Δ*icl2* deletion mutant. However, in work done in the MMRU, Savvi *et al.* (2008) subsequently demonstrated that the 3-NP-mediated growth inhibition of MTB on propionate could be alleviated by enabling the methylmalonyl pathway through addition of vitamin B_{12} to the growth medium (Savvi *et al.*, 2008). This result demonstrated the functionality of the methylmalonyl pathway *in vitro* and so identified a third option available to MTB for the detoxification of propionate. It also reinforced the idea that the dispensability of the methylcitrate pathway observed *in vivo* (Muñoz Elías *et al.*, 2006) could result from the functioning of the methylmalonyl pathway, and so confirmed the potential relevance of vitamin B_{12} to mycobacterial pathogenesis. The extent to which vitamin B_{12} availability dictates the functioning of this B_{12}-dependent pathway remains unclear; however, it does raise important questions pertaining to MTB's ability to access and/ or synthesize the cofactor *in vivo*. Previous studies have implicated other vitamins including vitamin B_5 (Sambandamurthy *et al.*, 2002), and vitamin B_6 (Dick *et al.*, 2010) in MTB pathogenesis. However, the metabolically demanding biosynthesis of a cofactor of the structural complexity of vitamin B_{12} in MTB remains poorly explored by comparison.

1.5 Vitamin B_{12}

Vitamin B_{12}, also known as cobalamin (Cbl), was first discovered when patients suffering from pernicious anaemia showed clinical as well as haematological improvements when they consumed a diet of raw liver (Whipple and Robscheit-Robbins, 1925; Minot and Murphy, 1926). This discovery earned Minot, Murphy and Whipple the Nobel Prize in Physiology and Medicine in 1934. Twenty years later, the anti-pernicious anaemia factor was independently isolated by Rickes *et al.* (1948) and Smith (1948) as a red crystalline compound from liver, and was named Vitamin B_{12}. Owing to its importance for human health, vitamin B_{12} has been extensively studied ever since.

1.5.1 Structure of vitamin B_{12}

In 1956, Dorothy Hodgkin and colleagues elucidated the complex, three-dimensional structure of vitamin B_{12} (Hodgkin *et al.*, 1956) and, together with Lenhert, later revealed the structure of the biologically active form of vitamin B_{12}, adenosylcobalamin (AdoCbl) (Lenhert and Hodgkin, 1961). This earned Hodgkin the second Nobel Prize for B_{12}-related research in 1964, this time in the field of Chemistry. Cbl belongs to the same structurally complex prosthetic group as chlorophyll, heme, siroheme, and coenzyme F_{430}, all of which are derived from the common macrocyclic biosynthetic intermediate, uroporphyrinogen III (Raux *et al.*, 1998a; Rodionov *et al.*, 2003). Structurally, Cbl is composed of a cobalt-centred corrin ring which is attached via an aminopropanol linker to a lower α-ligand - 5,6-dimethylbenzamidazole (DMB) - and to an upper β-ligand comprising a methyl (methylcobalamin; MeCbl), adenosyl group (AdoCbl), or hydroxyl group (OHCbl) (Figure 1.1) (Raux *et al.*, 1999). Naturally occurring analogues of vitamin B_{12} have either an adenosyl or methyl

group occupying the β-ligand: the adenosyl group is found in analogues involved in rearrangement or reductase reactions, while the methyl group is present in the cofactor that is involved in B_{12}-dependent methionine synthesis (Marsh, 1999). Vitamin B_{12}, or cyanocobalamin (CNCbl), is the industrially produced form that has a cyano group occupying the upper ligand as a result of the extraction procedure of the molecule from bacterial cultures (Martens *et al.*, 2002). This form can be converted to either of the biologically active forms, AdoCbl or MeCbl (Martens *et al.*, 2002). Vitamin B_{12} and its analogues are often termed corrinoids and therefore, the abbreviation B_{12}, will be utilized to refer to all forms of Cbl throughout this thesis.

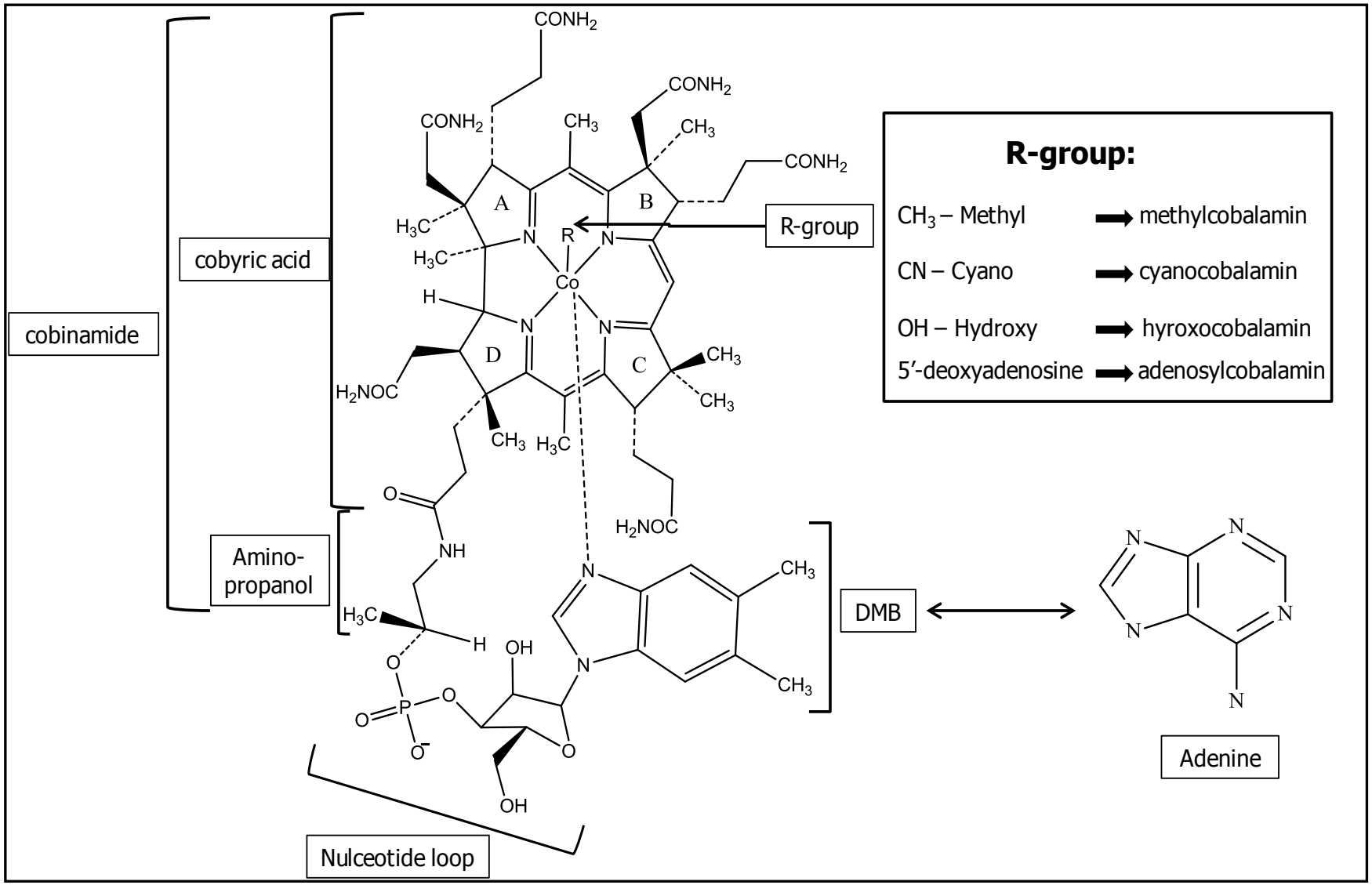
cobinamide
cobyric acid
Amino-
propanol
Nulceotide loop
R-group
DMB
Adenine
R-group:
CH3 – Methyl ➡ methylcobalamin
CN – Cyano ➡ cyanocobalamin
OH – Hydroxy ➡ hyroxocobalamin
5'-deoxyadenosine ➡ adenosylcobalamin

Figure 1.1: Structure of vitamin B_{12} and derivatives. Vitamin B_{12} (CNCbl) comprises a central corrin ring, a lower dimethylbenzimidazole (DMB) ligand, and an upper ligand containing a cyano group. CNCbl is the industrially produced form; the forms that occur in nature are methylcobalamin (MeCbl), hydroxocobalamin and adenosylcobalamin (AdoCbl; coenzyme B_{12}), in which the cyano group is replaced as upper ligand (Martens *et al.*, 2002; Warren *et al.*, 2002).

1.6 Vitamin B_{12} biosynthesis

Vitamin B_{12} is an essential nutrient required by various forms of life; however, the *de novo* biosynthesis of this molecule is restricted to a select group of bacterial and archaeal species (Martens *et al.*, 2002; Rodionov *et al.*, 2003). It has been proposed by Roth and colleagues (1996) that the B_{12} biosynthetic pathway originally evolved to allow anaerobic fermentation of small molecules in the absence of an electron acceptor and, as the pathway continued to evolve, siroheme (allowing use of inorganic electron acceptors), chlorophyll (oxygen production), and heme (aerobic respiration) were produced. As the atmospheric levels of oxygen increased, many organisms lost fermentative functions and metabolic dependency on B_{12} decreased (Roth *et al.*, 1996). However, both aerobic and anaerobic pathways for vitamin B_{12} biosynthesis have been identified in bacteria.

A diverse set of enzymatic reactions involving the products of more than 30 genes (denoted *cob* and *cbi* genes) is required for *de novo* biosynthesis of B_{12} (Roth *et al.*, 1993). Previously, AdoCbl biosynthesis was divided into three discrete steps: *CobI*, which entails the conversion of uroporphyrinogen III to the AdoCbl intermediate cobinamide; *CobII*, which involves the synthesis of DMB from probable flavin precursors; and *CobIII*, in which the covalent joining of cobinamide, DMB, and a phosphoribosyl group completes synthesis of the cofactor (Roth *et al.,* 1993; Lawrence

and Roth, 1996). However, this schematic was rejected when distinct routes for B_{12} biosynthesis were distinguished: an oxygen-dependent pathway characterized in *Pseudomonas denitrificans* (*cob* genes) (Stamford, 1994) and an oxygen-independent pathway characterized in *Salmonella typhimurium* (*cbi* genes) (Roth *et al.*, 1993). Instead, the identification of these pathways resulted in the reclassification of the pathway to comprise two major parts (Rodionov *et al.*, 2003). The first step is an energetically demanding process that involves corrin ring synthesis, and differs in the aerobic and anaerobic pathways in terms of cobalt insertion - that is, the two pathways diverge at the precorrin-2 step and merge again at adenosylcobyric acid (Raux *et al.*, 1999; Warren *et al.*, 2002). In oxygen-dependent synthesis, precorrin-2 is methylated to precorrin-3A; in contrast, in oxygen-independent synthesis, precorrin-2 is chelated with cobalt to give cobalt-precorrin-2 (Raux *et al.*, 1999). Therefore, these two pathways are quite distinct in that the oxygen-independent pathway begins with the insertion of cobalt, while in the oxygen-dependent pathway, insertion of cobalt occurs much later - after nine further reactions. In addition, the cobalt-chelatases utilized in these pathways differ: in the oxygen-dependent pathway, CobNST requires ATP, whereas the oxygen-independent chelatase from *S. typhimurium*, CbiK, does not require any high-energy metabolites (Raux *et al.*, 1997; Rodionov *et al.*, 2003). The second part of the synthetic pathway is common to both aerobic and anaerobic routes, and comprises adenosylation, attachment of aminopropanol, and assembly of the nucleotide loop (Warren *et al.*, 2002).

Besides differing in the timing of cobalt insertion and the requirement for molecular oxygen, the aerobic and anaerobic synthetic pathways can be distinguished at the genetic level according to the presence or absence of hallmark genes. For example, aerobic ring contraction requires two enzymes, CobG and CobJ, the former requiring molecular oxygen for

activity. From comparative genetic studies, there seems to be no homologue of this monoxygenase in facultative anaerobes such as *S. typhimurium* (Roth *et al.*, 1993) and *Propionibacterium shermanii* (Santander *et al.*, 1997). Instead, organisms which synthesize B_{12} anaerobically contain two other indispensable enzymes (Raux *et al.*, 1996; Raux *et al.*, 1998b): CbiD, which is involved in C-1 methylation (Roessner *et al.*, 2005), and CbiG, a cobalt-precorrin-5A hydrolase (Kajiwara *et al.*, 2006). The anaerobic CbiD has been suggested to be a homologue of the aerobic methyltransferase CobF (Roper *et al.*, 2000). Additionally, no homologues of *P. denitrificans* genes *cobNST* - which are required for late cobalt insertion - or *cobF*, *cobE* and *cobW*, were found in *S. typhimurium* (Raux *et al.*, 1996; Warren *et al.*, 2002). Conversely, *S. typhimurium cbiK*, which is required for cobalt chelation of precorrin-2, is not found in *P. denitrificans* (Raux *et al.*, 1997).

Although there appear to be certain hallmark features of the aerobic and anaerobic routes in terms of the presence or absence of specific genes, some bacteria have been shown to synthesize B_{12} under both conditions. Lawrence and Roth (1996) examined the B_{12} biosynthetic capability of enteric bacteria and found that most organisms belonging to this family are able to synthesize B_{12} both aerobically and anaerobically. However, *S. typhimurium* seems to be an exception in that it is only able to synthesize B_{12} *de novo* under strict anaerobic conditions (Jeter *et al.*, 1984). Perhaps to compensate, this bacterium is able to take up incomplete corrinoids and the α-ligand DMB aerobically to synthesize B_{12} (Escalante-Semerena *et al.*, 1990). Uptake of only cobinamide, an incomplete corrinoid composed of the corrin ring plus aminopropanol side chain, generates only 100 molecules of B_{12}, which is apparently insufficient to support growth on ethanolamine; however, uptake of both cobinamide plus DMB markedly increases B_{12} production (Andersson and Roth, 1989; Carkeet *et al.*, 2006). Another enteric bacterium, *E. coli*, lacks the bulk of the genes

required for *de novo* B_{12} biosynthesis (Lawrence and Roth, 1996). This bacterium appears to possess remnants of the B_{12} pathway that allow it to synthesize a complete molecule by attaching upper and lower ligands to incomplete corrinoids (Lawrence and Roth, 1995). Therefore, in these organisms, an incomplete set of biosynthetic genes is compensated for by uptake utilizing an elaborate transport system.

1.6.1 Synthesis of the lower ligand 5,6-dimethylbenzamidazole (DMB)

Until recently (Gray and Escalante-Semerena, 2007; Taga *et al.*, 2007), the synthesis of the α-axial ligand, DMB, was the one of the steps of the B_{12} biosynthetic pathway that remained a mystery. Previous studies demonstrated anaerobic synthesis of DMB in *Eubacterium limosum* from erythrose, glycine, formate, glutamine and methionine (Warren *et al.,* 2002); however, enzymes catalysing these reactions have not yet been identified (Escalante-Semerena, 2007; Taga and Walker, 2008). As with corrin ring synthesis, an aerobic DMB pathway was also elucidated which showed that DMB was derived from flavin mononucleotide (FMN) (Renz, 1970; Höllriegl *et al.*, 1982; Keck *et al.*, 1998). Recent studies on the aerobic pathway in *Rhodospirillum rubrum* and *Sinorhizobium meliloti* found that the *bluB* gene was necessary for the formation of DMB from FMN in an oxygen dependent manner (Campbell *et al.*, 2006; Gray and Escalante-Semerena, 2007; Taga *et al.*, 2007). This was further confirmed in *S. meliloti* by introducing point mutations in two key residues commonly observed in *bluB* homologues (Taga *et al.*, 2007). Mutation of aspartate at position 32 and the glycine at position 167 in the *S. meliloti* BluB, either individually or in combination, abrogated DMB synthesis. Interestingly, *Salmonella enterica* synthesizes the corrin ring anaerobically and derives DMB aerobically from FMN, yet no ortholog of *bluB* appears to exist in its

genome (Keck *et al.*, 1998; Anderson *et al.*, 2008). Bioinformatic analyses have revealed the *Rv0306* gene in MTB as a putative homologue of *S. meliloti bluB* (Rodionov *et al.,* 2003; Taga *et al.,* 2007). A crucial similarity between *S. meliloti* BluB and Rv0306 is the conservation of the two key residues described above.

1.6.2 Mycobacterial B_{12} biosynthesis

The genome sequence of the commonly utilized laboratory strain, MTB H37Rv, was published in 1998 (Cole *et al.*, 1998). Subsequent comparative bioinformatic analysis of genes involved in B_{12} biosynthesis has elucidated putative homologues in MTB (Figure 1.2), revealing that the organism possesses a near-complete B_{12} biosynthetic pathway (Rodionov *et al.*, 2003; Dawes, unpublished). Key features of the predicted pathway are suggestive of aerobic B_{12} biosynthesis in MTB: the putative mono-oxygenase, CobG, which contains an iron-sulphur centre and is responsible for converting precorrin-3A into precorrin-3B, is of the type that requires molecular oxygen for activity (Debussche *et al.*, 1993; Spencer *et al.*, 1993a). In addition, MTB possesses an aerobic-type CobN. Moreover, it is predicted that cobalt insertion occurs late in the MTB B_{12} biosynthetic pathway, and the MTB CobK and CobJ orthologues show conservation of residues commonly found in aerobic bacteria (Shearer *et al.*, 1999; Warren *et al.*, 2002). Interestingly, however, MTB appears to possess some features that are characteristic of anaerobic biosynthesis; for example, Rv0259c exhibits homology to CbiX, a cobalt chelatase (Raux *et al.*, 1998b) that was identified in *Bacillus megaterium* and shown to chelate Co^{2+} into sirohydrochlorin - an intermediate of heme biosynthesis that can either be utilized in the synthesis of siroheme or AdoCbl (Leech *et al.*, 2002; Raux *et al.*, 2003). The functionality of this homologue is yet to be proven in MTB. *Pseudomonas aeruginosa* also possesses hallmarks of

both oxygen-dependent (CobG, CobN) and -independent (CbiD, CbiG) pathways, and is able to synthesize B_{12} aerobically and anaerobically (Raux *et al.*, 2000). Therefore, the presence of these enzymes is consistent with the notion that MTB encounters oxygen-limiting environments *in vivo* to which it must adapt.

Interestingly, other mycobacteria including *Mycobacterium smegmatis* (MSM)*, M. bovis and Mycobacterium avium* also appear to have retained either all or parts of this pathway (Dawes, unpublished), with B_{12} biosynthetic genes in MSM (Figure 1.2) indicative of aerobic synthesis (Rodionov *et al.*, 2003; Dawes, unpublished). The predicted mycobacterial B_{12} biosynthetic pathways are consistent with results of a study by Karasseva *et al.* (1977) which used a microbiological assay to infer vitamin B_{12} biosynthesis by mycobacteria including *M. bovis*, *M. phlei* and MSM. This is despite the fact that no homologues of CobST - which in *P. denitrificans*, forms a complex with CobN and is responsible for cobalt insertion (Debussche *et al.,* 1992) - could be identified by homology searches in mycobacterial genomes. Cobalt chelatase activity is only observed in the presence of the two component system (Warren *et al.*, 2002). It is possible that insertion of cobalt in mycobacteria is mediated by CysG which, in *S. tyhphimurium*, has been demonstrated to act as both a ferrochelatase for siroheme synthesis and a cobaltochelatase for B_{12} biosynthesis (Fazzio and Roth, 1996). In *E. coli*, CysG is a multifunctional enzyme whose N-terminus is similar to that of CobA proteins which are responsible for methyltransferase activity, and whose C-terminus provides cobaltochelatase activity (Spencer *et al.*, 1993b; Warren *et al.*, 1994). MTB CysG exhibits homology to the *E.coli* protein over the entire length of the gene, including the NAD^+ binding site in the N-terminus. In MTB and *M. bovis*, no homologue was found for CobF, which functions as a deacetylase and a methyltransferase in *P. denitrificans* (Debussche *et al.*, 1993; Min *et al.*, 1993); however this enzyme is present in MSM

(*MSMEG_5548*) and *M. avium* (*MAV_1065*). In *M. bovis*, the 5' region of *cobL* has been deleted in addition to two upstream genes (Brosch *et al.*, 2002), yet this mycobacterium still appears to be able to synthesize B_{12} (Karasseva *et al.*, 1977). Based on sequence comparisons, the bifunctional activity of CobL is predicted to be in the N- and C-terminal regions, which encode the methyltransferase and decarboxylase activities, respectively (Roth *et al.*, 1993; Raux *et al.*, 2000). In contrast, the equivalent protein in *S. typhimurium* is encoded by two separate genes, *cbiT* and *cbiE* (Raux *et al.,* 2000; Rodionov *et al.,* 2003). Interestingly, *cbiE* shows homology to *cobI* in addition to *cobL* suggesting that *cobI* may be able to substitute for *cobL* in mycobacteria.

1.6.3 Vitamin B_{12} biosynthesis in MTB

Putative homologues for most of the B_{12} biosynthetic genes have been identified in MTB. This near-complete B_{12} biosynthetic pathway appears not to contain homologues of *cobF* and *cobST*. CobF functions as a methyltransferase (Min *et al.*, 1993) and, in MTB, a nonorthologous displacement of CobF is predicted to be encoded by *Rv2067c*, which houses a C-terminal methyltransferase domain (Figure 1.2) and has been designated as metZ (Rodionov *et al.*, 2003). Late-stage cobalt insertion into hydrogenobyrinic-acid a,c-diamide is catalyzed by three subunits: CobN, CobS and CobT (Debussche *et al.*, 1992). CobS and CobT are thought to form a complex that interacts with CobN to generate cob(II)yrinic acid a,c-diamide (Figure 1.2). The cobalt chelatase subunits CobN, CobS and CobT display significant homology to the magnesium chelatase subunits ChlH, ChlI and ChlD that function in bacteriochlorophyll biosynthesis (Gibson *et al.*, 1995; Schubert *et al.*, 1999). Therefore, it is hypothesized (Rodionov *et al.*, 2003) that ChlD is the missing component of the cobaltochelatase complex which, in MTB, is encoded by *Rv2850c*

and is annotated as a magnesium chelatase (Figure 1.2). Notably, the gene encoding the cob(II)yrinic acid a,c-diamide reductase in the aerobic B_{12} biosynthetic pathway is yet to be identified in any organism. Although a NADH-dependent flavoprotein exhibiting cob(II)yrinic acid a,c-diamide reductase activity was purified in *P. denitrificans* (Blanche *et al.*, 1992a). In MTB, *Rv0306* was predicted to encode the cobalt reductase function (Rodionov *et al.*, 2003). Despite the presence of the near-complete genetic repertoire for vitamin B_{12} biosynthesis, genetic evidence suggests that this pathway is not functional *in vitro* in MTB (Warner *et al.*, 2007; Savvi *et al.*, 2008). These observations appear to contradict the findings of Karasseva *et al.* (1977), and so demand further investigation.

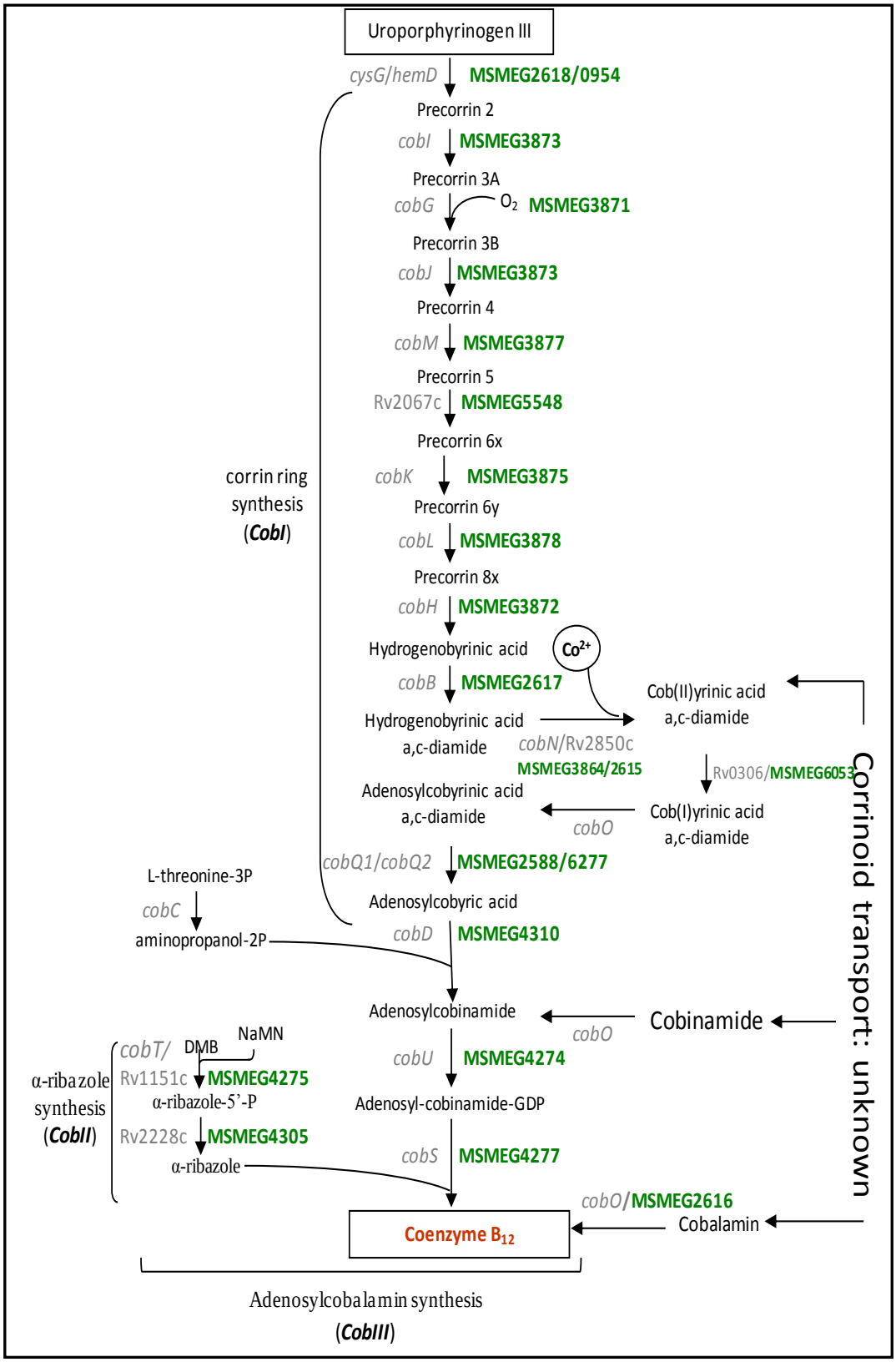
Uroporphyrinogen III
cysG/hemD
MSMEG2618/0954
Precorrin 2
cobI
MSMEG3873
Precorrin 3A
cobG
O2
MSMEG3871
Precorrin 3B
cobJ
MSMEG3873
Precorrin 4
cobM
MSMEG3877
Precorrin 5
Rv2067c
MSMEG5548
Precorrin 6x
cobK
MSMEG3875
Precorrin 6y
cobL
MSMEG3878
Precorrin 8x
cobH
MSMEG3872
Hydrogenobyrinic acid
Co2+
cobB
MSMEG2617
Hydrogenobyrinic acid a,c-diamide
cobN/Rv2850c
MSMEG3864/2615
Cob(II)yrinic acid a,c-diamide
Rv0306/MSMEG6053
Cob(I)yrinic acid a,c-diamide
cobO
Adenosylcobyrinic acid a,c-diamide
cobQ1/cobQ2
MSMEG2588/6277
Adenosylcobyric acid
cobD
MSMEG4310
corrin ring synthesis (CobI)
L-threonine-3P
cobC
aminopropanol-2P
Adenosylcobinamide
cobO
Cobinamide
Corrinoid transport: unknown
cobT/
DMB
NaMN
Rv1151c
MSMEG4275
α-ribazole-5'-P
Rv2228c
MSMEG4305
α-ribazole
α-ribazole synthesis (CobII)
cobU
MSMEG4274
Adenosyl-cobinamide-GDP
cobS
MSMEG4277
cobO/MSMEG2616
Cobalamin
Coenzyme B12
Adenosylcobalamin synthesis (CobIII)

Figure 1.2: Predicted pathway for AdoCbl biosynthesis in MTB. MTB H37Rv gene designations (http://genolist.pasteur.fr/Tuberculist/) are shown together with predicted *Mycobacterium smegmatis* mc^2155 (MSM) homologues in green (all MSM gene names have the underscore removed for simplification). The pathway is adapted from Roth *et al.* (1993), Rodionov *et al.* (2003) and S. Dawes (unpublished); putative MSM homologues were identified by BLAST homology search (Altschul *et al.*, 1990) of the preliminary MSM database (http://tigerblast.tigr.org/cmr.blast/) using MTB query sequence.

1.7 Alternate B_{12} cofactors: Pseudo-B_{12}

A spectrum of B_{12} analogues exists in nature whose functions are not yet fully understood. They share structural architecture with 'orthodox' B_{12} in the corrin ring with cobalt at the core and the β-axial ligand consisting of 5'-deoxyadenosine, or a methyl group, but with a different α-axial ligand (Figure 1.1). Substituting DMB with phenolic compounds or, in the most common example, the purine nucleobase, adenine, generates an alternate B_{12} form known as pseudo-B_{12} (Figure 1.1). First reported in 1952 by Pfiffer and colleges, pseudo-B_{12} was isolated from an organism in the bovine rumen and was shown to be an inadequate substitute for vitamin B_{12} in small mammals and humans (Berman *et al.*, 1956). However, *E. coli* mutants that responded to either methionine or B_{12} supplementation demonstrated the same response when supplemented with pseudo-B_{12} (Davis, 1952). This alternate B_{12} was later identified by UV and visible (UV-Vis) spectroscopy to be the native cofactor of *Clostridium tetannomorphum* (Barker *et al.,* 1958). Subsequently, this alternate form of vitamin B_{12} was found to be synthesized by a number of microorganisms such as *Propionibacterium shermanii* (Friedmann and Fyfe, 1969), *Clostridium sticklandii* (Stadtman, 1960), and *Lactobacillus reuteri* (Santos *et al.,* 2007).

1.7.1 Role of alternate B_{12} cofactors

More recently, Anderson and colleges (2008) demonstrated the ability of *S. enterica* to synthesize – and utilize – pseudo-B_{12}. Specifically, these authors showed that pseudo-B_{12} could be used by all the B_{12}- dependent enzymes of *S. enterica* raising the possibility that an "alternate" cofactor like pseudo-B_{12} might represent the preferred form under periods of high demand. Significantly, they also demonstrated that biosynthesis of pseudo-B_{12} from "incomplete" corrinoid precursors requires the same set of enzymes (CobT, CobU, CobS, and CobC) involved in the synthesis of "orthodox", DMB-containing vitamin B_{12}. This result indicated that nucleotide activation and attachment enzymes accommodate a variety of substrates. In addition, their results established that, *S. enterica* has the capacity to synthesize "complete" corrinoids aerobically by importing an incomplete corrinoid such as cobinamide and adding appropriate α- and β-axial ligands, but is also able to generate B_{12} or pseudo-B_{12} *de novo* under anaerobic conditions. Subsequently, it was shown that *Cyanobacterium synechocytis* is able to synthesize pseudo-B_{12} *de novo* and this cofactor is utilized by the B_{12}-dependent methionine synthase (*metH*) in this organism (Tanioka *et al.*, 2009). Together, these observations suggest that the formation and utilization of pseudo-B_{12} is a natural physiological process and, further, that environmental conditions (for example, oxygen availability) might dictate the form of the cofactor synthesized (and utilized).

The potential of pseudo-B_{12} to function in "classic" B_{12}-dependent metabolism holds significant implications for MTB pathogenesis as well as the role of "alternate" B_{12} forms in other mycobacterial pathogens. For example, comparative genomic analyses have identified key B_{12} biosynthetic and B_{12}-dependent genes, as well as active pathways for the biosynthesis and scavenging of purines (including adenine) in *M. leprae*

(Wheeler, 1987; Dawes and Mizrahi, 2001), a pathogenic mycobacterium that has undergone extensive reductive evolution and is thought to approximate a minimal mycobacterial gene set (Cole *et al.*, 2001). These observations raise questions as to what form of B_{12} might be utilized during host infection by MTB and other mycobacterial pathogens.

1.8 Vitamin B_{12}-dependent enzymes

Despite the versatility of the cofactor, the actual number of known B_{12}-dependent enzymes remains small (Raux *et al.*, 2000). Three classes of B_{12}-dependent enzymes are known: the isomerases, the methyltransferases, and the reductive dehalogenases (Banerjee and Ragsdale, 2003). Members of all three classes of B_{12}-dependent enzymes are important in microorganisms, as well as in human and animal metabolism; however, B_{12}-dependent processes are yet to be identified in plants, fungi and insects (Croft *et al.*, 2005). While mammals are restricted to two B_{12}-dependent enzymes - methionine synthase and methylmalonyl-CoA mutase - prokaryotes harbour a variety enzymes requiring B_{12} cofactors (Roth *et al.*, 1996; Banerjee and Ragsdale, 2003; Zhang and Gladyshev, 2009). For example, even though *E. coli* possesses relatively few B_{12} biosynthetic genes, it possesses four B_{12}-dependent enzymes: ethanolamine ammonium lyase, epoxyqueuosine reductase, methylmalonyl-CoA mutase and a B_{12}-dependent methionine synthase (Lawrence and Roth, 1996).

1.8.1 B_{12}-dependent enzymes in MTB

The MTB genome contains three enzymes which require vitamin B_{12} derived cofactors for activity. These enzymes are predicted to participate in different aspects of cellular metabolism. The first, a methylmalonyl-CoA mutase encoded by *mutAB* (Rainwater and Kolattukudy, 1985; Savvi *et al.,* 2008), serves to catalyze the conversion of (*R*)-methylmalonyl-CoA – generated from β-oxidation of fatty acids – to succinyl-CoA. Intermediates from this pathway contribute to integral cell wall lipids which are thought to play a major role in survival of the bacterium in hostile *in vivo* environments. As described above, this pathway could also potentially present another route for propionyl-CoA detoxification, provided that sufficient B_{12} is available to satisfy the requirements of the enzyme. In humans, methylmalonyl-CoA mutase is required for the degradation of odd-chain fatty acids and certain branched-chain amino acids (Ledley, 1990). Deficiency of the enzyme often results in fatal methylmalonoic acidemia as a result of the accumulation of propionyl-CoA and methylmalonyl-CoA (Allen *et al.*, 1993; Qureshi *et al.*, 1994).

The second enzyme in MTB that requires a vitamin B_{12}-derived cofactor is the B_{12}-dependent methionine synthase, encoded by *metH* (Warner *et al.*, 2007). This enzyme has been shown to be functional in certain strains of MTB and comprises four functional units: an N-terminal homocysteine-binding domain, methyltetrahydrofolate-binding domain, Cbl-binding domain and C-terminal S-adenosyl-L-methionine (SAM)-binding domain (Goulding *et al.*, 1997; Warner *et al.*, 2007). Interestingly, MetH in CDC1551 is truncated by 398 amino acids at the C-terminus, partially disrupting the Cbl-binding domain and completely eliminating the SAM-binding domain which renders the enzyme non-functional and the clinical isolate a natural *metH* mutant (Warner *et al.*, 2007). MetH catalyzes the transfer of a methyl group from N^5-methyl-tetrahydrofolate to the thiolate

of homocysteine, thereby generating the essential amino acid, methionine (Banerjee and Matthews, 1990). The same reaction is also catalyzed by MetE a B_{12}-independent methionine synthase that utilizes a different catalytic mechanism (Gonzalez *et al.*, 1992; Matthews *et al.*, 2003; Pejchal and Ludwig, 2004). Humans encode only the B_{12}-dependent enzyme, while organisms that are unable to transport or synthesize the cofactor, such as yeasts, fungi, and plants, encode only the B_{12}-independent enzyme (Hondorp and Matthews, 2004). However, many bacterial species that do not synthesize B_{12} *de novo* and some that synthesize B_{12} exclusively under anaerobic conditions (e.g. *S. typhimurium*), encode both methionine synthase enzymes (Pejchal and Ludwig, 2004). In *E. coli*, these enzymes are differentially expressed; that is, MetH is only expressed in the presence of B_{12} which also serves to repress MetE while, in the absence of B_{12}, MetE is the sole methionine synthase that is expressed (Greene, 1996). In humans, inhibition of methionine synthase results in the development of megaloblastic anaemia (Banerjee and Matthews, 1990; Allen *et al.*, 1993).

The last of the three vitamin B_{12}-dependent enzymes in MTB is a class II ribonucleotide reductase (RNR), encoded by *nrdZ* (Dawes *et al.*, 2003). Ribonucleotide reductases are responsible for converting the pool of ribonucleosides to deoxyribonucleosides and therefore play an essential role in DNA repair and replication (Sjöberg and Sahlin, 2001). There are three classes of RNRs: the first can be subdivided into class Ia, Ib and Ic and require oxygen to generate radicals for catalysis; the second class (including NrdZ) does not require oxygen but is dependent on AdoCbl as a cofactor for radical generation; while the third class contains anaerobic enzymes that are inactivated by oxygen and generate a glycyl radical from S-adenosylmethionine and an iron-sulfur cluster (Jordan and Reichard, 1998; Högbom *et al.*, 2004; Nordlund and Reichard, 2006). MTB possesses a class Ib RNR which comprises *nrdE*-and *nrdF2*-encoded

subunits and has been shown to be essential under *in vitro* conditions (Dawes *et al.*, 2003). Interestingly, the MTB genome also contains two other putative class Ib RNR small subunits NrdF1 and NrdB, but these are dispensable for growth *in vivo* and *in vitro* under various stresses (Mowa *et al.*, 2009). The class II RNR is encoded by *nrdZ* which is part of the DosR/DevR regulon (Dawes *et al.*, 2003; Voskuil *et al.*, 2004b). In a mouse model of TB infection, a Δ*nrdZ* mutant displayed no *in vivo* growth phenotype, suggesting that the putative class II RNR does not play a role during infection, at least in the murine model (Dawes *et al.*, 2003).

Superficially, none of the B_{12}-dependent functions in MTB appears to justify *de novo* synthesis of the cofactor. At least in the case of NrdZ and MetH, corresponding B_{12}-independent enzymes are predicted to catalyze similar functions: that is, the MTB genome encodes a class Ib RNR encoded by *nrdE* and *nrdF2* (Dawes *et al.*, 2003) and a B_{12}-independent methionine synthase encoded by *metE* (Warner *et al.*, 2007). However, it has been shown that, in *E. coli*, the reaction catalyzed by vitamin B_{12}-dependent MetH is 100-fold faster than the reaction catalyzed by the vitamin B_{12}-independent version, MetE (Greene, 1996). Furthermore, the B_{12}-independent enzymes, NrdZ and MetH, have been shown to be non-essential for growth of MTB *in vitro* (Dawes *et al.*, 2003; Warner *et al.*, 2007). In the case of MutAB, dispensability of this enzyme is suggested by the alternate detoxification mechanisms of propionyl-CoA that exist in MTB (as discussed in section 1.4.3). These results are intriguing since they suggest redundancy of all three B_{12}-dependent enzymes; however, it is possible that enzyme multiplicity signals the importance of the different pathways to the survival of the bacterium. That is, differential enzyme utilization might be demanded of MTB by the heterogeneous environments encountered during colonization of the human host (Warner and Mizrahi, 2006).

1.9 Bacterial B_{12} regulation

Vitamin B_{12} is known to repress the expression of genes required for its own biosynthesis and transport (Vitreschak *et al.*, 2003). For example, the full *cob* operon that encodes the B_{12} biosynthetic genes of *S. typhimurium*, and the *btuB* genes in *E. coli* and *S. typhimurium* which encode the B_{12} outer membrane transporter, are all repressed by exogenous B_{12} via a post-translational regulatory mechanism (Lundrigan *et al.*, 1991; Richter-Dahlfors and Andersson, 1992; Vitreschak *et al.*, 2003). In addition, in bacteria that possess both B_{12}-dependent and B_{12}-independent enzymes, activity of the B_{12}-independent enzyme is often subject to regulation by B_{12} (Vitreschak *et al.*, 2003). Regulation occurs through the activity of B_{12} riboswitches - mRNA structural elements (Vitreschak *et al.*, 2003) that serve as ligand-responsive genetic controls to modulate the expression of certain genes in response to changing concentrations of metabolites (Nahvi *et al.*, 2004). Typically, riboswitches are embedded within the 5'-untranslated region of the mRNA sequence and are composed of two functionally distinct domains (Nahvi *et al.*, 2002; Santillán and Mackey, 2005). The aptamer domain provides a ligand binding pocket that binds the target metabolite with high selectivity (Mandal and Breaker, 2004). The other domain is an expression platform which is usually located downstream of the aptamer domain and controls the expression of adjacent genes or operon by harnessing allosteric changes in the RNA structure brought about by the aptamer-metabolite conformation (Nahvi *et al.*, 2002; Barrick and Breaker, 2007). Vitamin B_{12}-sensing riboswitches are among the largest, with aptamer domains of about 200 nucleotides (nt) to accommodate this large metabolite (Gruber *et al.*, 2011). The first direct evidence of riboswitches as regulatory elements was obtained utilizing a technique called in-line probing (Nahvi *et al.*, 2002), which takes advantage of the spontaneous degradation of RNA due to internal transesterifications (Regulski and Breaker, 2008a).

Nahvi and colleagues (2002) demonstrated that the *E. coli btuB* mRNA leader sequence can bind directly to AdoCbl, resulting in conformational changes in the secondary and tertiary structure of the RNA. The possible mechanism of regulation involves the formation of two alternate RNA structures, repressing and anti-pressing, in the presence or absence of B_{12} respectively (Nahvi *et al.*, 2002).

In a recent study, Gallo *et al.* (2008) used in-line probing to demonstrate the interaction of the *E. coli btuB* aptamer with various derivatives of B_{12}. The 202 nt riboswitch is situated in the 5'-UTR of the *btuB* gene which encodes an outer membrane protein used for transport of B_{12} derivatives. The riboswitch underwent conformational change upon binding to AdoCbl, CNCbl, adenosyl factor A, and adenosyl-cobinamide (AdoCbi) provided that the ligand concentrations were high enough. This observation suggested that rather than the α- or β- ligands of the B_{12} molecule, the corrin ring was responsible for triggering the structural change of the aptamer domain (Gallo *et al.*, 2008).

The MTB genome contains two putative B_{12} riboswitch motifs which are located immediately upstream of the *metE* and *PPE2* genes, respectively (Rodionov *et al.,* 2003; Vitreschak *et al.,* 2003). The riboswitch upstream of *metE* has been shown to regulate transcription of the B_{12}-independent methionine synthase (*metE*) in response to increased B_{12} levels (Warner *et al.*, 2007). Importantly, this study, as well as the related work by Savvi *et al.* (2008) revealed that MTB does not produce B_{12} under standard conditions *in vitro*. In addition, these publications also provided the first direct evidence of vitamin B_{12} transport in MTB (Warner *et al.*, 2007).

1.10 B_{12} transport in bacteria

Owing to its large size, B_{12} is unable to pass through the outer membrane porins (Nikaido, 1994) and, therefore, is actively transported across the outer and cytoplasmic membranes with the aid of highly effective and elaborate B_{12} uptake systems. The only known transport systems for B_{12} in bacteria are the *btuBFCD* systems in Gram-negative bacteria and the *btuFCD* system in Gram-positive bacteria (Vitreschak *et al.*, 2003). In enteric bacteria including *E. coli*, vitamin B_{12}, cobinamide, and other corriniods are actively transported using the TonB-dependent outer member receptor BtuB which forms a complex with the ABC transport system BtuFCD (Cadieux *et al.*, 2002; Cadieux *et al.*, 2003). BtuFCD belongs to a large superfamily involved in the uptake of iron, siderophores, and heme (Zhang and Gladyshev, 2009). Corriniods present in femtomolar (fM) concentrations in the environment (Bassford Jr and Kadner, 1977) are taken up by the BtuB protein which is a Ca^{2+}-dependent transporter located in the outer membrane (Heller *et al.*, 1985; Bradbeer *et al.*, 1986; Cherezov *et al.*, 2006). Delivery of the corriniods to the periplasmic space by BtuB requires energy provided through interactions with TonB, which is located in the inner membrane (Ferguson *et al.*, 2007; Postle and Larsen, 2007). Once in the periplasmic space, corrinoids are bound by the BtuF protein (Van Bibber *et al.*, 1999; Cadieux *et al.*, 2002) which facilitates delivery to the ABC transporter BtuCD - itself located in the inner membrane (DeVeaux and Kadner, 1985; Borths *et al.*, 2005) - in an ATP-dependent manner (Gruber *et al.*, 2011).

Based on comparative bioinformatic analysis, there appear to be no homologues of the *E. coli* B_{12} transport system components in MTB. The complete or partial lack of these *btu*-type B_{12}-specific transporters in MTB is surprising given the demonstration by Warner *et al.* (2007) that the MTB Δ*metE* mutant was able to grow when supplemented with B_{12}. For

this reason, and notwithstanding the absence of any supporting evidence, the presence of a B_{12} riboswitch directly upstream of *PPE2* is at least strongly suggestive of the involvement of the gene in some aspect of B_{12} or B_{12}-dependent metabolism. For example, genes possessing B_{12} riboswitch motifs in other bacteria include B_{12} or cobalt transporters, chelatases, and corrin ring methyltransferases (Rodionov *et al.*, 2003). Interestingly, Rodionov *et al.* (2003) identified *PPE2* as a putative B_{12}-regulated cobalt transporter that these authors termed CbtG. Notably, *PPE2* appears to lie in a putative operon with two B_{12} biosynthetic genes, consistent with the observation that most B_{12}-regulated genes are either found in B_{12} gene clusters or are scattered along the chromosome (Vitreschak *et al.*, 2003). Recently, *S. meliloti* was shown to encode an ABC-type transport system possessing a B_{12} riboswitch in the 5'UTR that transports cobalt, and not Cbl (Cheng *et al.*, 2011). Similarly, genes of the energy-coupling factor (ECF) cobalt transporter encoded by CbiMNQO in *Rhodobacter capsulatus*, are often co-localized with B_{12} biosynthetic genes or under the control of a B_{12} riboswitch (Rodionov *et al.*, 2006; Zhang and Gladyshev, 2009).

1.10.1 Cobalt transporters in bacteria

Cobalt is utilized at very low concentrations but plays an important role in biological systems where it is predominantly incorporated into the corrin ring of the B_{12} molecule and in non-corrin cobalt-dependent enzymes (Komeda *et al.*, 1997). Similar to the transport of B_{12}, cobalt ions must be transported across the outer membrane into the periplasmic space, where they can be transported through the inner membrane into the cytosol. As noted above, this process can occur via an ABC transporter like the *S. meliloti* CbtJKL cobalt transporter (Cheng *et al.*, 2011), or the CbiMNQO cobalt transporter found in *Rhodobacter capsulatus* (Rodionov *et al.,*

2006). Alternatively, nickel-cobalt (NiCoT) permeases can facilitate transport of cobalt (Komeda *et al.*, 1997). Cobalt can also be taken up via non-specific metal transport systems; for example, CorA proteins are generally associated with transport of magnesium ions but some members of the CorA family have also been shown to transport cobalt and nickel (Niegowski and Eshaghi, 2007; Zhang and Gladyshev, 2009).

1.11 The PE and PPE multigene families

The genome sequence of MTB contains two highly polymorphic sets of genes belonging to the PE and PPE families. These acidic, glycine rich proteins consume 10% of the coding capacity of the MTB genome (Cole *et al.*, 1998) and have attracted considerable interest in the TB research field as their biological functions have not yet been fully elucidated (Sampson, 2011). The PE/PPE genes are abundant in pathogenic mycobacteria such as MTB, *M. bovis*, *M. ulcerans*, *M. Kansasii*, and *M. marinum*, which is suggestive of a role in pathogenesis (Van Pittius *et al.*, 2006; Bottai and Brosch, 2009). Furthermore, the non-pathogenic MSM - whose genome is considerably larger than MTB (7Mb versus 4Mb) - contains only two pairs of PE and PPE genes (Van Pittius *et al.*, 2006).

The names "PE" and "PPE" are derived from the presence of N-terminal proline-glutamate or proline-proline-glutamate motifs, respectively (Cole *et al.*, 1998). Even though the PE/PPE genes appear to be scattered throughout the genome of MTB, detailed analysis has revealed that they are in fact arranged in organized operons. A total of 40 operons have been identified with 22 of these containing specific PE/PPE gene pairs, in which a PE coupled with a PPE gene (Strong *et al.*, 2006; Tundup *et al.*, 2006). The remaining operons contain either a PE or PPE gene, with other genes including conserved hypotheticals, metabolic genes or ESAT-6

genes. It is notable, therefore, that *PPE2* is located in a putative operon with B_{12} biosynthetic genes namely, *cobU* and *cobQ1*.

The N-terminal domains are conserved within the respective PE and PPE families, whereas the C-terminal domains of both families are of variable size and sequence (Van Pittius *et al.*, 2006). This has enabled the families to be divided into subfamilies based on sequence homology and characteristic motifs in their C-terminal domains. The PE family is composed of 99 members that have ~110 amino acids conserved in the N-terminal domains (Cole *et al.*, 1998). The polymorphic GC-rich repetitive sequence (PGRS) subfamily is the largest within the PE family and is composed of 65 members with multiple tandem repeats of glycine-glycine-alanine (Gly-Gly-Ala) or glycine-glycine-asparagine (Gly-Gly-Asn) motifs in the C-terminal domains (Cole *et al.*, 1998; Van Pittius *et al.*, 2006). The remaining subgroups of the PE family consist of proteins with C-terminal domains of low homology.

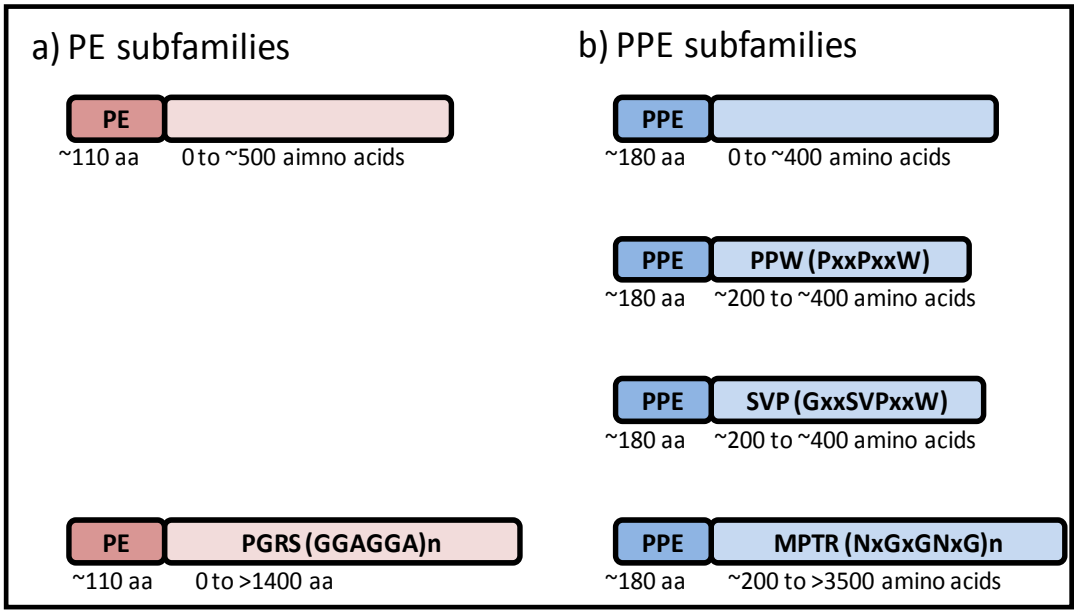

Figure 1.3: Diagrammatic representation of the PE and PPE subfamilies in MTB. The PE (a) and PPE (b) subfamilies possess conserved N-terminal domains of approximately 110 and 180 amino acids respectively, and variable C-terminal domains which allow classification of proteins into various subfamilies. X represents any amino acid. Adapted from van Pittius *et al.* (2006).

The PPE family is made up of 68 members that have ~180 amino acids in the N-terminal domains and are divided into four subfamilies. The PPE-SVP subfamily is the largest, with 24 members that are characterized by a conserved Gly-X-X-Ser-Val-Pro-X-X-Trp motif between position 300 and 350 in the amino acid sequence (Adindla and Guruprasad, 2003; Van Pittius *et al.*, 2006). The major polymorphic tandem repeats (MPTR) subfamily is the second largest, and consists of 23 members. The PPE-MPTR subfamily is characterized by multiple repeats of Asn-X-Gly-X-Gly-Asn-X-Gly in the C-terminal and is encoded by a consensus repeat sequence GCCGGTGTTG that is spaced by 5bp nucleotides (Hermans *et al.*, 1992; Cole and Barrell, 1998). The third PPE subfamily consists of 12 members that display low homology at the C-terminus (Van Pittius *et al.*, 2006). The final subfamily, PPE-PPW, consists of only 10 members and is

characterized by 44 amino acid residues at the C-terminus comprising of highly conserved Gly-Phe-X-Gly-Thr and Pro-X-X-Pro-X-X-Trp motifs (Adindla and Guruprasad, 2003). PPE2 falls within the PPE-PPW subfamily (Figure 1.3).

1.11.1 Localisation and potential function of PE/PPE proteins

Although it has been more than a decade since their discovery, the biological function of the PE/PPE proteins remains elusive (Sampson *et al.*, 2001). Of the 169 PE/PPE genes analysed in one study, 128 were shown to be differentially regulated under 15 different conditions (Voskuil *et al.*, 2004b). In addition, some PE/PPEs have been shown to be required for *in vivo* survival (Ramakrishnan *et al.*, 2000; Sassetti *et al.*, 2003; Singh *et al.*, 2008) suggesting their importance in infection. In a study by Brennan *et al.* (2001), it was demonstrated that a transposon mutant of PE-PGRS33 (encoded by *Rv1818c*) was associated with dispersed growth in liquid medium and impaired ability to enter and survive within macrophages, suggesting a role for this protein in macrophage uptake. Further studies on PE-PGRS33 have implicated this protein in host cell apoptosis (Balaji *et al.*, 2007; Basu *et al.*, 2007) and necrosis of macrophages (Dheenadhayalan *et al.*, 2006). In another study, PPE18 (*Rv1196*) was shown to interact with TLR2 receptors on macrophages and activate IL-10 production, which has been shown to inhibit the host immune response and thereby promote replication and persistence of MTB inside the host cells (Redpath *et al.*, 2001; Nair *et al.*, 2009). Other PE/PPEs have been implicated in phagosome maturation arrest (Hestvik *et al.*, 2005) and modulation of vacuole acidification (Li *et al.*, 2005; Jha *et al.*, 2010). Therefore, there appears to be growing evidence of a possible role for these proteins in interacting with host immune components, thereby altering and even enabling the bacterium to avoid innate immune

pathways. Consistent with this idea, studies have shown that PE/PPEs are cell wall-associated (Banu *et al.*, 2002; Okkels *et al.*, 2003; Le Moigne *et al.*, 2005) or even partially exposed on the cell surface (Brennan *et al.*, 2001; Sampson *et al.*, 2001) thereby allowing them to be secreted from the bacterium into the host cell. Further evidence of their surface exposure includes the observation that 40 PE/PPE proteins have β-barrel signatures, which are found in surface-exposed proteins having membrane-anchored regions (Pajon *et al.*, 2006). Thus, they appear to be ideally positioned to interact with the host immune system. In fact, many studies have demonstrated that these proteins can elicit an immune response; for example, a humoral immune response was detected in infected mice against the PGRS domain of PE-PGRS33, and a cell-mediated immune response was detected against the PE domain (Delogu and Brennan, 2001). Similarly, mice immunized with the PE25 and PPE41 pair showed increased T-cell proliferation and higher levels of interferon gamma, tumor necrosis factor alpha, and IL-2 cytokines (Tundup *et al.*, 2008). Serology studies carried out by Espitia *et al.* (1999) and Singh *et al.* (2001), demonstrated antibodies against PE-PGRS81 (Rv1759c) and the PGRS domain of Rv3367, respectively, in sera of TB infected patients, suggesting that some proteins from this family are present during infection.

M. marinum appears to have the largest number of PE/PPE genes (15 and 106 respectively) (Stinear *et al.*, 2008), of which two PE_PGRS genes were shown to be required for survival and persistence in macrophages, again supporting the idea that these proteins are virulence factors (Ramakrishnan *et al.*, 2000). Other functions in which this family has been implicated include the up-regulation of a PPE37 gene (Rv2123) under low iron conditions, suggesting that this gene may encode a siderophore that is involved in iron uptake (Rodriguez *et al.*, 1999; Rodriguez *et al.*, 2002). Similarly, the PE_PGRS protein encoded by Rv1759c was found to display

fibronectin-binding properties (Espitia *et al.*, 1999). Additionally, PE-PPE11 (LipX) and PE_PGRS63 (LipY) demonstrated lipase activity (Deb *et al.*, 2006; Mishra *et al.*, 2008), while PE_PGRS11 also exhibited phosphoglycerate mutase activity (Chaturvedi *et al.*, 2010).

Owing to the technical difficulties experienced when working with PE/PPE proteins, only one co-crystal structure has been solved thus far, that of the PE25/PPE41 complex (Strong *et al.*, 2006). The structure revealed that the most conserved regions of the PE/PPE pair were responsible for protein-protein interactions, and also identified the presence of a possible docking site, which in turn suggested that other proteins could be recruited to the complex (Strong *et al.*, 2006). Further analysis revealed that the PPE protein of the complex shared some features with a serine chemotaxis receptor; therefore, it was proposed that PE/PPE complexes may be involved in host immune sensing and signalling (Strong *et al.*, 2006). More structural data are clearly necessary in order to understand the function of these proteins. Overall, this intriguing family has been implicated in disease pathogenesis and other non-related areas; however complete understanding of their function remains a research priority.

1.11.2 Association of PE/PPEs with esx regions

Comparative genomic studies have revealed that the evolution and expansion of the *PE/PPE* gene families are coupled with the *esx* regions (van Pittius *et al.*, 2001). The MTB genome contains five *Esx* gene clusters which encode the components of the Type VII or ESX secretion systems (van Pittius *et al.*, 2001). The five ESX systems (ESX-1 to ESX-5) in MTB display a highly conserved gene organization that comprises a pair of *esx* genes in the centre of the cluster, accompanied by *PE/PPE* genes upstream and then flanked by membrane proteins and components of an

ATP-powered secretion machine (DiGiuseppe Champion and Cox, 2007; Bottai and Brosch, 2009). The best characterized of these is the ESX-1 system, which is known to secrete the 6-kDa early secreted antigenic target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10), encoded by *esxA* and *esxB*, respectively (Sørensen *et al.*, 1995; Berthet *et al.*, 1998). These highly immunogenic proteins have been shown to modulate TLR2 signalling and cytokine inhibition (Sørensen *et al.*, 1995; Berthet *et al.*, 1998; Skjot *et al.*, 2000; Pathak *et al.*, 2007). The ESX-1 system is located in the region of difference-1 (RD1), which is absent in the attenuated *M. bovis* BCG vaccine strain (Mahairas *et al.*, 1996). The importance of the RD1 region in virulence has been established experimentally by deleting this region in H37Rv and confirming that it resulted in attenuation of the strain; conversely, incorporation of this region into the attenuated BCG vaccine strain resulted in expression of ESAT-6 and CFP-10 and increased virulence (Pym *et al.*, 2002; Lewis *et al.*, 2003; Pym *et al.*, 2003). A functional ESX-1 system is required for the export of ESAT-6 and CFP-10, which have been demonstrated to be required for mycobacterial spread to uninfected macrophages (Guinn *et al.*, 2004), inhibition of macrophage inflammatory response (Stanley *et al.*, 2003), cell lysis and tissue invasion (Hsu *et al.*, 2003).

In the MTB genome, the ESX-2 locus is located downstream of ESX-1; however, relatively few studies have focused on this system, or on ESX-4 which represents the most ancestral system. As a result, nothing is presently known about their functions (van Pittius *et al.*, 2001; Stinear *et al.*, 2008). Genes in the ESX-3 cluster were shown to be regulated by iron and zinc in MTB (Maciag *et al.*, 2007), suggesting involvement of this system in homeostasis of these metal ions. The faster growing MSM possesses ESX-1, ESX-4 and ESX-3 only (van Pittius *et al.*, 2001), of which the latter cluster was shown to respond to iron and not zinc (Maciąg *et al.*, 2009). In a study utilizing MSM and *M. bovis*, it was demonstrated that the

ESX-3 secretion system responds to iron deprivation and is required for infection of macrophages (Siegrist *et al.*, 2009). In addition, the ESX-3 system was shown to be essential for growth in liquid medium using a tetracycline (Tet)-inducible conditional mutant (Serafini *et al.*, 2009). The repression phenotype was reversed by addition of zinc, iron or supernatant from the parental strain, which led the investigators to conclude that ESX-3 might encode a novel metal uptake system.

The final ESX system, ESX-5, has been studied in *M. marinum*. Abdullah *et al.* (2006) were the first to demonstrate a link between this system and a PPE protein. They showed that an ESX-5 mutant of *M. marinum* was unable to secrete the heterogeneously expressed MTB PPE41 protein; moreover, introduction of the ESX-5 cluster into MSM resulted in the secretion of PPE41 (Abdallah *et al.*, 2006) confirming the involvement of the machinery encoded by this system in the secretion of the PPE protein. In addition, the ESX-5 system was found to be involved in modulating the immune response by suppressing proinflammatory cytokines and inducing 1L-1β production (Abdallah *et al.*, 2008). This PE-PGRS and PE-MPTR secretion pathway was also shown to be involved in suppressing cytokine secretion in a TLR-dependent manner (Abdallah *et al.*, 2008; Abdallah *et al.*, 2009). Although ESX-5 was shown to be a major secretion system for some PE and PPE proteins, the possibility that other PE/PPE proteins might be secreted via other systems including other ESX systems cannot be ruled out. In addition, this information was deduced in *M. marinum*; while these results might apply in MTB, this link is yet to be demonstrated formally.

1.12 Mycobacterial features examined in this thesis

The biosynthesis of B_{12} is complex and requires an intricate pathway comprising 30 sequential enzymatic steps. For this reason, it is intriguing that MTB is included in a select group of microorganisms that appear to possess a near-complete biosynthetic pathway. Previous work in the MMRU has characterized B_{12}-dependent metabolic processes (Dawes *et al.*, 2003; Warner *et al.*, 2007; Savvi *et al.*, 2008). Amongst other findings, these studies surprisingly indicated that MTB does not synthesize B_{12} *de novo* despite possessing a near-complete biosynthetic pathway (Warner *et al.*, 2007; Savvi *et al.*, 2008). In addition, bioinformatic analysis revealed that there is a complete lack of typical B_{12} transport systems in the MTB genome, a result that is inconsistent with the ability of exogenous B_{12} to complement multiple growth phenotypes in MTB (Warner *et al.*, 2007; Savvi *et al.*, 2008). In addition, the presence of a B_{12} riboswitch upstream of *PPE2*, and the suggestion by Rodionov *et al.* (2003) that this gene might be a putative B_{12}-regulated cobalt transporter, stimulated our interest in the potential role for PPE2 in B_{12} or cobalt transport in MTB. Furthermore, taking into account the versatility of MTB, we also sought to investigate whether MTB could synthesize and utilize alternate B_{12} cofactors such as pseudo-B_{12} in B_{12}-dependent metabolic processes.

1.13 Aims

This study was initially directed towards investigating a role for PPE2 in B_{12} metabolism in MTB, and to ascertaining the capacity of B_{12} precursors to function in B_{12} biosynthesis in mycobacteria. To this end, the project originally comprised two primary components:

1. An investigation of the role of MTB PPE2 (Rv0256c) in vitamin B_{12} or cobalt transport.
2. An analysis of the ability of substrates like vitamin B_{12} (CNCbl), the B_{12} precursors cobinamide, DMB, adenine and cobalt to complement growth of MTB and MSM vitamin B_{12} auxotrophs.

Subsequent observations in this study indicated that *PPE2* did not have a role in B_{12} transport in MTB; however, its importance in either B_{12} synthesis or cobalt transport still existed owing to the tantalising presence of a B_{12} riboswitch which indicated the likely B_{12}-dependent regulation of *PPE2* function. During the course of this project, it also became evident that MTB might be able to synthesize B_{12} *de novo* under certain *in vitro* conditions. Moreover, key questions arose pertaining to the precise form of B_{12} and B_{12}-derived cofactors that mycobacteria utilize. The original aims of the project were therefore updated and expanded to include the following set of major aims that formed the basis of this dissertation:

1. An analysis of the ability of substrates including vitamin B_{12} (CNCbl), the B_{12} precursors cobinamide, DMB, adenine and cobalt to complement growth of MTB and MSM vitamin B_{12} auxotrophs already constructed within the laboratory (Warner *et al.*, 2007; Dawes, unpublished).
2. An investigation of the role of MTB PPE2 (Rv0256c) in cobalt transport or assimilation.
3. An analysis of the ability of mycobcateria to synthesize and utilize pseudo-B_{12} as a functional equivalent of vitamin B_{12} in key B_{12}-dependent metabolic pathways. Abrogation of DMB synthesis by

eliminating the putative *bluB* (Rv0306) formed a core component of this aim as it enabled the genetic differentiation of vitamin B_{12} biosynthesis (and utilisation) from the synthesis of pseudo-B_{12}.

2. Materials and Methods

All general procedures and DNA manipulations were performed according to standard protocols (Sambrook and Russell, 2001).

2.1 Bacterial strains and culture conditions

All bacterial strains utilized in this study were stored at -70°C in 30% (v/v) glycerol and are detailed in Table 2.1.

- ***Escherichia coli* DH5α**

Escherichia coli was cultured in Luria-Bertani broth (LB) at 37°C overnight with vigorous shaking (Labcon Shaking Incubator) or at 30°C for 48 h (New Brunswick Scientific Innova 400 incubator shaker), supplemented with appropriate antibiotics where necessary. Luria Bertani agar (LA) plates supplemented with appropriate antibiotics where necessary was used to culture *E. coli* containing plasmids, which were incubated at 37°C overnight (Incotherm Labotec Incubator) or at 30°C for 48 h (Heraeus Instrument Incubator). Strains carrying large plasmids (≥8000bp) were incubated at 30°C to minimise the risk of plasmid rearrangements. For selection purposes the following antibiotics were used at the indicated concentrations: ampicillin (Amp), 200μg/ml; hygromycin (Hyg), 100-200 μg/ml; and kanamycin (Km), 50 μg/ml. For negative selection of clones that carry the *sacB* gene, 5% (w/v) sucrose was used. For positive selection of clones and/or to confirm disruption of the *lacZ* for cloning purposes, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 20 mg/ml in deionised dimethyl formamide) was utilized.

Table 2.1: General strains used in this study

Strain	Description	Reference
Escherichia coli		(Snapper *et al.*, 1990)
DH5α	*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Promega
Mycobacterium smegmatis		
mc^2 155	High-frequency transformation mutant of *M. smegmatis ATCC 607*	(Snapper *et al.*, 1990)
Δ*metE*::*hyg*	*metE* deletion mutant in mc^2 155; Hyg^R	(Dawes, unpublished)
Δ*cobK*	*cobK* deletion mutant in mc^2 155	(Dawes, unpublished)
Δ*cobK* Δ*metE*::*hyg*	Double *metE*|*cobK* deletion mutant in mc^2 155	(Dawes, unpublished)
Mycobacterium tuberculosis		
H37Rv	Virulent laboratory strain ATCC 25618	Laboratory collection
Δ*PPE2*	*PPE2* deletion mutant of H37Rv	This study
Δ*metE*::*hyg*	*metE* deletion mutant of H37Rv; Hyg^R	(Warner *et al.*, 2007)
Δ*cobK* Δ*metE*::*hyg*	double *metEcobK* deletion mutant of H37Rv; Hyg^R	D. F. Warner, unpublished
Δ*PPE2* Δ*metE*::*hyg*	marked double *metE*|*PPE2* deletion mutant of H37Rv; Hyg^R	This study
Δ*cobU* Δ*metE*::*hyg*	marked double *metE*|*cobU* deletion mutant of H37Rv; Hyg^R	D. F. Warner, unpublished
Δ*metE*::*hyg* Δ*PPE2attB*::*PPE2*	Δ*PPE2* complemented with full-length *PPE2* at *attB* site; Hyg^R, Km^R	This study
Δ*metE*::*hyg*Δ*PPE2 attB*::*PPE2-cobQ1-cobU*	Δ*PPE2* complemented with entire *PPE2-cobQ1-cobU* locus; hyg^R, Km^R	This study
Δ*metE*::*hyg*Δ*cobU attB*::*PPE2-cobQ1-cobU*	Δ*metE*::*hyg*Δ*cobU* complemented with entire *PPE2-cobQ1-cobU* locus ; Hyg^R, Km^R	This study
Δ*Rv0306*	*Rv0306* deletion mutant of H37Rv	This study
Δ*metE*::*hyg* Δ*Rv0306*	Double *metE* |*Rv0306* deletion mutant of H37Rv; Hyg^R	This study
$\Delta Rv0306^{D32NS167G}$Δ*metE*::*hyg*	*metE* with site-directed point mutations in *Rv0306*;Hyg^R	This study

- ***Mycobacterium smegmatis***

Unless otherwise stated, MSM strains were cultured in Middlebrook 7H9 liquid medium (Difco) supplemented with 0.1% $Tween_{80}$, 0.2% glycerol and a glucose- salt solution (GS; 0.085% NaCl, 0.2% glucose). For growth on solid media, 7H10 Middlebrook (Difco) supplemented with 0.5% glycerol and glucose-salt (GS; 0.085% NaCl, 0.2% glucose) was used. Cultures were grown in Erlenmeyer flasks at 37°C shaking (Incotherm Labotec Incubator). Where necessary, antibiotics were used at the following concentrations: Km, 10 µg/ml or 25 µg/ml; and Hyg, 50 µg/ml. Unless otherwise indicated, the following supplements and cofactor precursors were used at the indicated concentrations: adenine (Sigma), 10 µM; cobinamide (dicyanocobinamide, Sigma), 10 µM; 5,6-dimethylbenzamidazole (Sigma), 10 µM; and vitamin B_{12} (cyanocobalamin, Sigma), 10 µg/ml (8 µM).

- ***Mycobacterium tuberculosis***

Unless indicated, MTB strains were grown in Middlebrook 7H9 liquid medium (Difco) supplemented with 0.05% $Tween_{80}$ and 0.2% glycerol, or on 7H10 Middlebrook solid medium (Difco) supplemented with 0.5% glycerol. 100 ml oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) was added to both liquid and solid media (10% v/v). Culturing and manipulation of MTB strains was carried out in a biosafety level 3 laboratory in a Class II flow cabinet at negative pressure of at least 160 kPa. Cultures were grown at 37°C in tissue culture flasks that were placed flat or in roller bottles. For negative selection of clones that carry the *sacB* gene, 2% (w/v) sucrose was used. For positive selection of clones harbouring the *lacZ* gene, β-galactosidase activity (blue-white selection) was assessed by adding X-gal (Roche) at a standard concentration of 20 mg/ml in deionised dimethyl sulfoxide (DMSO; Sigma) in 25 ml agar plates. Antibiotics were used at the following concentrations where

necessary: Km, 10 µg/ml or 25 µg/ml; and Hyg, 50 µg/ml. Unless otherwise indicated, the following supplements and cofactor precursors were used at the indicated concentrations: adenine, 10 µM; cobinamide, at 10 µM; 5,6-dimethylbenzamidazole (Sigma), 10 µM; 3-nitropropionate (3-NP; Sigma); 10 µM and Vitamin B_{12}, 10 µg/ml (8 µM).

2.2 Cloning vectors

All plasmids utilized in this study are detailed in Table 2.2.

Table 2.2: Plasmids used in this study

Strain	Description	Reference
p2NIL	*E. coli* cloning vector and mycobacterial suicide vector; Kn^R	(Parish and Stoker, 2000)
pGEM3Z(+)f	*E. coli* cloning vector; Amp^R, *lacZ*	Promega
pGOAL17	Plasmid carrying *lacZ* and *sacB* genes as a PacI cassette; Amp^R	(Parish and Stoker, 2000)
pGOAL19	Plasmid carrying *lacZ*, *hyg* and *sacB* genes as a PacI cassette; Amp^R	(Parish and Stoker, 2000)
p2Δ*cobKU19*	*cobK* knockout vector containing *Pac*I cassette from pGOAL19; Kn^R, Hyg^R, suc^S	K. Downing
p2*PPE2*	P2NIL containing *ΔPPE2* allele; Kn^R	This study
p2*PPE2U17*	*PPE2* knockout vector containing *PacI* cassette from pGOAL17; Kn^R, Hyg^R, suc^S	This study
p2*PPE2U19*	*PPE2* knockout vector containing *PacI* cassette from pGOAL19; Kn^R, suc^S	This study
p2Δ*Rv0306*	p2NIL containing *ΔRv0306* allele; Kn^R	This study
p2Δ*Rv0306U17*	*Rv0306* knockout vector containing *PacI* cassette from pGOAL17; Kn^R, Hyg^R, Suc^S	This study
p2Δ*Rv0306U19*	*Rv0306* knockout vector containing *PacI* cassette from pGOAL19; Kn^R, suc^S	This study
p2Rv0306^{D32NS167G} U17	p2NILcontaining *Rv0306* with site-directed point mutations; Kn^R, Hyg^R, suc^S	This study
pMC*PPE2*comp	PPE2 complementation vector- pMC1r carrying full length *PPE2* ; Kn^R	This study
pMC*PQU*comp	Complementation vector- pMC1r carrying entire *PPE2-cobQ1-cobU* locus ; Km^R	This study

2.3 DNA extraction

2.3.1 Plasmid extraction and purification from E.coli

- **Small-scale plasmid DNA isolation**

Stationary-phase cultures were harvested by centrifugation in an Eppendorf 5415D microcentrifuge (15000 x g for 1 min) at room temperature in 1.5 ml microfuge tubes. The supernatant was discarded and the pellet resuspended in 100 µl of lysis solution I (0.5 M glucose, 50 mM Tris·HCl pH 8.0, 10 mM EDTA); thereafter, 200 µl of solution II (0.2 M NaOH, 1% SDS) was added and the suspension was mixed gently. After 5 min of incubation at room temperature, 150 µl of neutralization solution III (3 M potassium acetate, pH 5.5) was added and gently mixed before centrifuging at 9000 x g for 5min at room temperature. The supernatant was transferred to fresh microfuge tubes and treated with 1 µl of 10 µg/ml RNase A (Sigma) for 10 min at 37°C. Plasmid DNA was precipitated by adding 350 µl of isopropanol, then incubated for 10 min at room temperature and collected by centrifugation at 15000 x g for 10 min. The pelleted DNA was washed with 70% ice-cold ethanol and dried at 45°C in a vacuum centrifuge (MiVac DNA concentrator, GeneVac). The DNA was resuspended in 20-30 µl of sterile distilled water (sdH_2O).

- **Large-scale plasmid DNA extraction**

Cultures were grown overnight in 50 ml of LB and harvested by centrifugation in a Beckmann J2-21 centrifuge (1100 x g for 10 min). Thereafter the extraction method was exactly as described for the small-scale extraction (above) except that the solution volumes were increased by a factor of 10. Once extracted and washed, the DNA was resuspended in a final volume of 500 µl of sdH_2O and precipitated by adding $1/10^{th}$ volume 5.3 M sodium acetate (pH 5.3). The DNA was purified by adding

equal volumes of phenol:chloroform (1:1; v/v) solution, the suspension was mixed vigorously and then centrifuged at 15000 x g for 10 min at room temperature. The aqueous phase was added to an equal volume of chloroform: isoamyl alcohol (24:1; v/v), mixed vigorously, and centrifuged at 15000 x g for 10 min at room temperature. The aqueous phase was removed and the plasmid DNA was further precipitated by adding 2.5 volumes of 100% ice-cold ethanol and incubating the solution at -20°C for 30 – 45 min. DNA was collected by centrifugation at 15000 x g for 10 min at room temperature, and the pellet was washed with 70% ice cold ethanol, then dried in a vacuum centrifuge and resuspended in 50-200 µl sdH_2O.

2.3.2 Chromosomal DNA extraction from mycobacteria

- ***Mycobacterium smegmatis***

Chromosomal DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB; ICN Biomedicals, Aurora, Ohio) method (Larsen, 2000). Briefly, cells were heat-killed in microfuge tubes for 20 min at 65°C, then harvested by centrifugation at 13000 x g for 1 min. After resuspending the pellet in 500 µl TE buffer (10 mM Tris·HCl pH 8.0, and 1 mM EDTA), 50 µl of lysozyme (10 mg/ml) was added and the reaction incubated overnight at 37°C. The following morning, 70 µl of 10% SDS and 6 µl of proteinase K (10 mg/ml) was added and incubated at 37°C for 2 h. Thereafter, 80 µl of pre-warmed CTAB/NaCl mix (10% CTAB made in 0.7 M NaCl) and 100 µl of 5M NaCl was added and the mixture was further incubated for 10 min at 65°C. Subsequent to incubation, an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added to remove residual proteins. The aqueous phase was added to an equal volume of isopropanol and then put on ice for 30 min. After centrifugation for 20 min

at 9000 x g, the pellet was washed with ice cold 70% ethanol and dried in a vacuum centrifuge before resuspending in 100 µl of sdH_2O.

- ***Mycobacterium tuberculosis***

The method used for MTB DNA extraction was similar to that described above except for minor differences and that all procedures prior to the overnight lysozyme step were carried out in a biosafety level 3 laboratory in a class II flow cabinet at negative pressure of 160 kPa. Briefly, cells were heat killed in microfuge tubes for an hour at 80°C, then harvested by centrifugation at 13000 x g for 1 min. After resuspending the pellet in 500 µl of TE buffer (10 mM Tris·HCl pH 8.0, and 1 mM EDTA), 50 µl of lysozyme (10 mg/ml) was added and this was incubated overnight at 37°C. Subsequent to incubation, 70 µl of 10% SDS and 50 µl of proteinase K (10 mg/ml) were added and this was incubated at 60°C for 1 h in a Thermomixer Compact (Eppendorf) set to low shaking mode (300 rpm). One hundred µl of pre-warmed CTAB/NaCl (10% CTAB, 0.7 M NaCl) and 100 µl 5 M NaCl was added, mixed thoroughly and then returned to 60°C for 15 min in the Thermomixer. Thereafter, microfuge tubes were placed at -70°C for 15 min, then removed and allowed to thaw before incubating at 60°C in the Thermomixer for a further 15 min. An equal volume of chloroform:isoamylalcohol (24:1) was added and the mixture was inverted several times to mix, then harvested by centrifugation at 9000 x g for 10 min. The aqueous phase was added to an equal volume of ice-cold isopropanol, mixed by inversion, and then stored at 4°C overnight. The following morning, chromosomal DNA was harvested by centrifugation at 9000 x g for 10 min, the pellet was washed with 70% ice-cold ethanol and dried in a vacuum centrifuge before resuspending in 55 µl of sdH_2O.

- **DNA extraction from mycobacteria and *E. coli* for PCR screening using the colony boil method**

Bacterial colonies were picked from agar plates and resuspended in 200 µl of TE buffer (10 mM Tris·HCl pH 8.0, 0.1 mM EDTA) before being heat-killed at 95°C for 5 min. An equal volume of chloroform was added, and the solution centrifuged at 15000 x g for 5 min. The aqueous phase was carefully extracted into fresh microfuge tubes and 2 µl was used for PCR.

2.4 Agarose gel electrophoresis

Standard electrophoretic techniques were applied when separating DNA fragments (Sambrook *et al.*, 1989; Sambrook and Russell, 2001). High molecular weight DNA fragments were separated on agarose gels of 0.8%-1% or 2%-4% agarose gels for low molecular weight fragments (≤1kb), all made in 1x TAE buffer (40 mM Tris-acetic acid, 1 mM Na_2EDTA pH 8.0). DNA samples were loaded with tracking dye (0.025% bromophenol blue in 30% glycerol) into gels that contained 0.5 µg/ml ethidium bromide. Fragment sizes were assessed using lambda DNA molecular weight markers (BMII-BMVI; Roche). Agarose gels were electrophoresed in a Mini-Sub Cell GT mini gel horizontal submarine unit (Bio-Rad) between 80-100 volts and visualized under UV-light using the Gel Doc 2000 system (Bio-Rad).

2.4.1 Recovery of DNA from agarose gels and quantification

The required DNA fragments were excised from agarose gels and purified using the Nucleospin Extract II Kit (Macherey-Nagel) as per the manufacturer's instructions. After purification, the eluted DNA was quantified either on an agarose gel by comparison to DNA molecular

weight markers, or on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

2.5 DNA manipulations

All DNA manipulations and molecular biology techniques were carried out according to standard protocols (Sambrook *et al.*, 1989).

- **Restriction enzyme digests**

All restriction enzymes were obtained from Amersham Pharmacia Biotech, New England Biolabs Inc., or Roche. All restriction enzyme digests were performed at 37°C unless otherwise required by the manufacturer. Plasmid DNA of up to 1 µg was digested between 1-3 h at 37°C (unless otherwise required by the manufacturer) with the appropriate reaction buffer(s). Mycobacterial DNA of up to 5 µg was digested overnight at 37°C (unless otherwise required by the manufacturer) with the buffer(s). Digested DNA fragments were separated and analysed on agarose gels (as detailed in sections 2.4 & 2.4.1).

- **Dephosphorylation**

Linearized plasmid DNA was treated with Antarctic Alkaline Phosphatse (New England Biolabs) to ensure removal of the 5'-phosphate and thereby prevent vector re-ligation. Dephosphorylation was performed for 1 h at 37°C after which the enzyme was heat inactivated for 20 min at 65°C (as per the manufacturer's instructions).

- **Ligation reactions**

Ligation reactions were carried out using either the Fast-link™ ligation kit (Epicentre ® Biotechnologies) or T4 DNA ligase (Roche) as per the manufacturer's instructions. Ligations were then transformed into *E. coli* DH5α cells as detailed in section 2.7.1.

2.6 Polymerase Chain Reaction (PCR)

Screening and preliminary PCRs were carried out using FastStart Taq (Roche) as per the manufacturer's instructions. Briefly, 20-50 µl reactions containing between 10-100 ng of plasmid or genomic DNA were set up with 1x reaction buffer, 200 µM of each dNTP, 0.5-1.0 µM of each primer, 1.5 mM $MgCl_2$, 1x GC-rich solution, and 2U/50 µl of DNA polymerase. Thermal cycler parameters used for DNA amplification were as follows: denature at 95°C for 4 min, followed by 30 cycles of denaturation (95°c for 30 s), annealing (60°C for 30 s), extension (72°C for 60s/kb), and a final extension at 72°C for 7 min. Fragments used for cloning purposes were amplified with Phusion High-Fidelity DNA polymerase (Finnzymes), which has a low error rate of 4.4 $x10^{-7}$. As per the manufacturer's instructions, 20-50 µl reactions contained: 1x reaction buffer, 200 µM of each dNTP, 0.5 µM of each primer, 3% DMSO or 1x GC rich solution, 0.02 U/µl of DNA polymerase and between 10-100 ng of plasmid or genomic DNA. The DNA amplification parameters were: initial denaturation at 98°C for 3 min, followed by 30 cycles of denaturation (98°C for 10 s), annealing (60°C for 30 s), extension (72°C for 30s/kb), and final extension at 72°C for 7 min. All PCR reactions were carried out in the MyCycler™ thermal cycler (Bio-Rad) with oligonucleotide primers (Table 2.4 to 2.8) purchased from Inqaba Biotech Ltd. or Integrated DNA Technologies (IDT).

2.7 Bacterial transformation

2.7.1 Chemical transformation of E. coli

- **Preparation of competent cells**

All competent cells were prepared using the rubidium chloride method obtained from Dr P. Stolt. Briefly, 1 ml of an overnight culture of *E. coli* DH5α was inoculated into 100 ml LB and grown to an OD_{600} of between 0.48-0.55. The cells were kept on ice for 15 min then harvested by centrifugation at 3901 x g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 20 ml transformation buffer (Tfb) I solution (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, and 15% v/v glycerol – pH 5.8). The suspension was chilled on ice for 15 min, and then harvested by centrifugation at 3901 x g for 5 min at 4°C. The pellet was then resuspended in 2 ml of TfbII solution (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride and 15 % v/v glycerol – pH 6.5), and 500 µl aliquots were flash-frozen in ice cold ethanol and stored at -80°C until required.

- **Transformation of *E. coli* with plasmid DNA**

The competent cells were thawed on ice, and 100 µl incubated with up to 1 µg of pre-chilled DNA in microfuge tubes on ice. After 20 min, the cells were heat-shocked at 42°C for 90 s then chilled on ice for 1-2 min; thereafter, 4 volumes of 2TY (Tryptone Yeast broth) was added to rescue the cells at 37°C for 1 h. The cells were plated on LA plates containing appropriate antibiotics and incubated at 37°C overnight (Sambrook *et al.*, 1989) or for two days at 30°C for constructs larger than 8 000 bp.

2.7.2 Transformation of mycobacteria

Mycobacteria were transformed by electroporation, which was performed according to previously published protocols, and as described briefly below (Larsen, 2000; Gordhan and Parish, 2001).

- **Electroporation into *Mycobacterium tuberculosis***

Briefly, 1 ml of a log-phase pre-culture was inoculated into 75 ml of 7H9/OADC media and grown to an OD_{600} 0.8-1.0. At least 6 h before competent cells were prepared, glycine (30%; w/v) was added to a final concentration of 1.5% (w/v). Cells were harvested by centrifugation at 1100 x g for 10 min at room temperature and the pellet was resuspended in 10% glycerol (w/v). Thereafter, cells were washed twice in 10% glycerol (w/v) then resuspended in 2-5 ml of 10% glycerol (w/v). Four hundred µl of competent cells were mixed with 2-5 µg of plasmid DNA in electroporation cuvettes (0.2 cm electrode gaps, Bio-Rad) and pulsed once in a GenePulser™ set at 2.5 kV, resistance 1000 Ω, capacitance 25 µF. Subsequent to electroporation, cells were rescued in 1 ml of 7H9/OADC then incubated overnight at 37°C. The following day, cells were spread on 7H10/OADC plates containing appropriate antibiotics and/or supplements where necessary and then incubated at 37°C for 21-28 days.

2.8 Southern blot analysis

- **Electroblotting**

Genomic DNA (2-5 µg) was digested overnight with appropriate restriction enzymes, separated by electrophoresis on a 0.8% agarose gel at 80 V (Section 2.4), and photographed with a ruler using the GelDoc 2000 system (Bio-Rad). The DNA was depurinated by immersing the agarose

gels in 0.25 M HCl for 10-12 min, then denatured by soaking in 1.5 M NaCl/0.5M NaOH solution for 25 min, and then neutralized (1.5 M NaCl, 5 M Tris·HCl, pH 7.5) for 30 min. Thereafter, the agarose gels were overlaid with Hybond™-N^+ membrane (Amersham), and sandwiched in a TE 22 Transphor cassette (Hoefer Scientific) between two pre-soaked 3 mm Whatmann (Merck) filter papers and two pre-soaked sponges. The cassette was placed in a TE 22 mini transfer unit (Hoefer Scientific) containing 1x Tris-Borate-EDTA (TBE) buffer (Sigma) and the DNA was transferred for 4 h at 0.4 A at 4°C. Once transferred, the DNA was cross-linked to the membrane by irradiation in a UV Stratalinker 1800 (Stratagene) at 1200 mJ/cm^2, and membranes were either hybridized immediately or wrapped in saran wrap and stored at 4°C until required.

- **Synthesis and labelling of probes**

The probes (detailed in Table 2.3) utilized for Southern blots were synthesized by PCR using primers detailed in Table 2.4 or by restriction enzyme digests, and then labelled according to the ECL Direct Nucleic Acid Labelling and Detection System protocol (Amersham). Briefly, a maximum of 100 ng of probe DNA in 10 µl of dH_2O was denatured by boiling for 5 min at 95°C, and immediately cooled on ice for 5 min. Subsequently, equivalent volumes of DNA labelling agent (Amersham) and glutaraldehyde solution (Amersham) were added and mixed gently. The resulting mixture was incubated at 37°C for 10 min and then used immediately in hybridization experiments.

Table 2.3: Probes used in southern blot analysis

Gene	Probe description
PPE2	860bp PCR product containing 170bp 3' *PPE2* coding sequence, generated using primer pair *PPE2*Fi/*PPE2*R1
metE	1740 bp PCR product containing 562 bp 5' *metE* coding sequence, generated using primer pair *metE*FR2
cobK	1162 bp *Xho*I/*Sal*I fragment from p2Δ*cobK*19
cobU	1750 bp PCR product containing 113 bp 3' *cobU* coding sequence, generated using primer pair *cobU*FR2
Rv0306	811bp PCR product containing 224bp 5' coding sequence, using primer pair *Rv0306*Fiseq/Riseq

- **Hybridization**

The hybridization buffer was prepared according to the ECL Direct Nucleic Acid Labelling and Detection System protocol (Amersham). Briefly, to 20 ml of hybridization buffer, 5% w/v blocking agent and 0.5 M NaCl was added and this was stirred at room temperature for 1 h and then at 42°C for 1 h. Subsequent to cross-linking, the membrane was pre-hybridized in roller bottles in the Hybridisation oven/shaker SI3OH (Stuart) for 1 h at 42°C, thereafter the labelled probe was added and this was hybridized overnight at 42°C.

Table 2.4: Oligonucleotides used to synthesize Southern blot probes

Name	Sequence (5'-3')
PPE2 Fi	CAGGGTGCCGTACACCCT
PPE2 R1	AAGGAGCGGGATCCCTGG
metE F2	GGGGGCCGGATCCAACTCTTCGAG
metE R2	GCGCGGGAAGCTTCAACTTCGGGCA
cobU F2	GGGGCGGGATCCCATTCTTCGGGCGT
cobU R2	GGCGGCGGTACCAAGGGCGAGCTGAAGT

- **Detection**

Following overnight hybridization, the membrane was washed in 5x SSC for 5 min at 42°C, then in primary wash buffer (6 M Urea, 0.4% SDS, 0.5x SSC) for 20 min at 42°C. The membrane was washed twice for 10 min in primary wash buffer at 42°C, and then twice for 5 min at room temperature in secondary wash buffer (0.5x SSC). Thereafter, detection reagents 1 and 2 (Amersham) mixed in equal quantities were overlaid over the membrane and incubated for 1 min at room temperature. The membrane was then drained, saran wrapped and exposed to X-ray film (Amersham Biosciences) in a cassette for time periods ranging from 1 min to 24 h at room temperature before developing.

2.9 Sequencing

All sequencing was performed as a pay-for-service by Inqaba Biotech Ltd, or the Central Analytical Sequencing Facility at Stellenbosch University.

2.10 Construction of MTB deletion mutants and complemented derivatives

- **Construction of *PPE2* deletion mutant of H37Rv**

The suicide vector used to generate the *PPE2* deletion was constructed using primer pairs *PPE2*F1/*PPE2*R1 and *PPE2*F2/*PPE2*R2 (Table 2.5) that amplified 1400 bp upstream (*PPE2*FR1) and 1686 bp downstream (*PPE2*FR2) of *PPE2* from H37Rv genomic DNA. Ligation of the resulting PCR products resulted in elimination of 1100 bp of the 1671 bp *PPE2* gene sequence when cloned into the *Asp*718/*Hind*III-digested p2NIL (Parish and Stoker, 2001), to generate p2Δ*PPE2*. Thereafter, the 7939 bp *Pac*I marker cassette from pGOAL19 (Parish and Stoker, 2001) containing *hyg*-

lacZ-sacB genes was cloned into the corresponding sites of p2Δ*PPE2* to generate p2Δ*PPE2*U19. This suicide vector was electroporated into H37Rv and the resulting Δ*PPE2* mutant was isolated by a two-step selection method as previously described (Gordhan and Parish, 2001). Genomic DNA from Δ*PPE2* was digested with *Sal*I and used in Southern blot analysis with the probe listed in Table 2.3 to confirm the genotype.

Table 2.5: Oligonucleotides used to construct knockout vectors used in this study

Name	**Sequence (5'-3')**	**Comments★**
PPE2 F1	GGCCACATGGTACCGAACA	1400bp sequence with naturally-occurring *Asp*718 site and introduced *Bam*HI site in reverse primer sequence
PPE2 R1	AAGGAGCGGGATCCCTGG	
PPE2 F2	GGCCTGGATCCACATCCG	1686bp sequence with introduced *Bam*HI site in forward primer sequence
PPE2 R2	CGCCGGTGAAGCTTGATCT	
Rv0306 F1	CTGCGTTGAAGCTGCAGGT	1599bp sequence with introduced *Psf*I site in forward primer sequence
Rv0306 R1	CGAGAATGCCCTCGACCTT	
Rv0306 F2	CCCGCGCTGGAACTGGAT	Contains naturally-occurring *Eco*RI (after forward primer) and *Asp*718 restriction sites
Rv0306 R2	GGTCACTGTGCGGTGACAA	

★The underlined sequence represents incorporated restriction sites

- **Generation of *PPE2* knockout in Δ*metE*::*hyg* mutant background**

The 6359-bp *PacI* marker cassette from pGOAL17 (Parish and Stoker, 2000) containing *lacZ-sacB* genes was cloned into the corresponding sites of the p2Δ*PPE2* vector (described above) to generate p2Δ*PPE2*U17. This suicide vector was electroporated into the hygromycin-resistant Δ*metE* mutant (Warner *et al.*, 2007) and the resulting double Δ*PPE2* Δ*metE*::*hyg* was phenotypically isolated by a two-step selection method previously described (Gordhan and Parish, 2001). Genomic DNA from the Δ*PPE2* Δ*metE*::*hyg* double mutant was digested with *Sal*I and used in Southern blot analysis with the probe listed in Table 2.3 to confirm the genotype.

- **Construction of Δ*Rv0306* knockout in MTB (H37Rv)**

Two primer sets, *Rv0306*F1/*Rv0306*R1 and *Rv0306*F2/*Rv0306*R2 were designed for PCR amplification of the upstream and downstream fragments of the *Rv0306* gene. The PCR products resulting from amplification of H37Rv genomic DNA - *Rv0306*FR1 (1599 bp) and *Rv0306*FR2 (1404 bp) - eliminated 313 bp of the 672 bp *Rv0306* gene sequence. Using a three way cloning strategy, these PCR products were cloned into the relevant sites of suicide vector p2NIL to create the p2Δ*Rv0306* construct. Thereafter, the 7939 bp *Pac*I marker cassette from pGOAL19 (Parish and Stoker, 2000) containing *hyg-lacZ-sacB* genes was cloned into the corresponding sites of p2Δ*Rv0306* to generate p2Δ*Rv0306*U19. The resulting suicide vector was electroporated into H37Rv and the Δ*Rv0306* mutant isolated by two-step selection as previously described (Gordhan and Parish, 2001). Genomic DNA from the Δ*Rv0306* was digested with *Stu*I and used in Southern blot analysis with the probe listed in Table 2.3 to confirm the genotype.

- **Construction of *Rv0306* knockout in the Δ*metE::hyg* mutant background**

The 6359 bp *PacI* marker cassette from pGOAL17 (Parish and Stoker, 2000) containing *lacZ-sacB* genes was cloned into the corresponding sites of the p2Δ*Rv0306* vector (described above) to generate p2Δ*Rv0306*U17. This suicide vector was electroporated into the hygromycin-marked Δ*metE* mutant (Warner *et al.*, 2007) and the resulting double mutant, Δ*Rv0306* Δ*metE*::*hyg* was phenotypically isolated by a two-step selection method previously described (Gordhan and Parish, 2001). Genomic DNA from the Δ*PPE2* Δ*metE*::*hyg* was digested with *Stu*I and used in Southern blot analysis with the probe listed in Table 2.3 to confirm the genotype.

- **Introduction of point mutations into *Rv0306* by site-directed mutagenesis**

A panel of synthetic genes comprising the entire MTB *Rv0306* sequence with specific point mutations was synthesized by Sigma-Aldrich®. The alleles were provided in an ampicillin-resistant vector, pG04v. The engineered mutations in Rv0306 included D32N and G167S; the synthetic sequences were also designed to include *Fsp*I and *Stu*I sites, both of which were used for rapid screening of putative transformants by restriction digest (detailed below). The pG04v vector was digested with *Asp*718 and the synthetic gene plus 336 bp of flanking sequence was cloned into the corresponding *Asp*718 site of p2NIL to create the p2*Rv0306*^{D32NS167G}*U17*. Thereafter, the *Pac*I marker cassette from pGOAL17 (Parish and Stoker, 2000) containing the *lacZ-sacB* genes was cloned into the corresponding sites of to generate p2*Rv0306*^{D32NS167G}*U17*. The resulting suicide vector was electroporated into Δ*metE*::*hyg*. Mutants were screened and confirmed by PCR and restriction enzyme digests (detailed below).

- **Screening of *Rv0306* point mutations**

To aid in the screening process, each of the point mutations was engineered so as to introduce an artificial restriction site: the D32N mutation introduced an *Fsp*I site, and the G167S mutation introduced a *Stu*I site. The screening process utilized was therefore two-fold: in the first step, the *Rv0306* gene was amplified using the primer pair *Rv0306*Frs/*Rv0306*Rrs (Table 2.6), which generated a 854 bp fragment that included 672 bp of the gene together with upstream (190 bp) and downstream (50 bp) sequence. Then, in the second step the PCR product was digested with *Fsp*I and *Stu*I, and positive transformants that harboured the point mutations generated 203 bp and 651 bp from the *Fsp*I digest; 597 bp and 257 bp from the *Stu*I digest.

Table 2.6: Oligonucleotides used in screening of *Rv0306* point mutations in this study

Name	Sequence (5'-3')	Comments
Rv0306 Frs	CTACTCGACTAGCCCGTGA	PCR product includes Rv0306 plus 190bp upstream and 50bp downstream
Rv0306 Rrs	CGTGCCGGCCGAATACGT	

- **Complementation of Δ*PPE2* mutant with the entire *PPE2* gene**

The *PPE2* gene was amplified from genomic DNA using the *PPE2*compF/*PPE2*compR primer pair (Table 2.7). The resulting 2495 bp PCR product, which contained the entire H37Rv *PPE2* plus 600 bp of upstream sequence, was digested with *Eco*RV and cloned into the blunted *Xmn*I site of the pMC1r integrating vector (Ahidjo *et al.*, 2011), generating the Km resistant pMC*PPE2*comp complementation vector. The

complementation vector was electroporated into the Δ*PPE* Δ*metE*::*hyg* double mutant and transformants selected on 7H10/OADC/B_{12} kanamycin plates. Putative complemented mutants were screened by PCR using *attB* primers (Barichievy, S., MSc dissertation, University of the Witwatersrand, 2005) to confirm site-specific integration at the *attB* chromosomal locus.

Table 2.7: Oligonucleotides used to construct complementation vectors

Name	Sequence (5'-3')	Comments★
*PPE2*compF	GCAGACCCGATATCCGTCT	2495bp with introduced *EcoRv* site in forward primer; a naturally-occurring *EcoRv* site is located downstream of the reverse primer
*PPE2*compR	GCCCGACAATGCAGCGCT	
*PQU*compF	CGACGATCCGCGCGTTTAAATGCCGGTGTA	4580bp with *DraI* introduced in forward and reverse primer
*PQU*compR	GGTCGCGGATATTTAAATGAGTAAGTCCTA	

★The underlined sequence represents a restriction site

- **Complementation utilizing the putative *PPE2-cobQ1-cobU* operon**

A second strategy to complement *PPE2* deletion entailed the introduction of the entire *cobU-cobQ1-PPE2* (PQU) operon at the *attB* locus. The putative operon was amplified using the *PQUcompF*|*PQUcompR* primer pair (Table 2.7) which generated a 4580 bp PCR product that included all three genes and 688 bp of upstream sequence. The PCR product was digested with *Dra*I and cloned into the relevant site of the pMC1r (Ahidjo *et al.*, 2011) integrating vector, generating the Km resistant pMC*PQU*comp construct. The complementation vector was electroporated into Δ*PPE2*

Δ*metE*::*hyg* and Δ*cobU* Δ*metE*::*hyg*, then selected on 7H10/OADC/B_{12} Km plates. Transformants were screened for site-specific integration at the *attB* chromosomal locus with *attB* primers (Barichievy, S., MSc dissertation, University of the Witwatersrand, 2005).

2.11 Validation of B_{12} and pseudo-B_{12} precursors utilizing *S. enterica*

The ability of *S. enterica* to utilize exogenously-supplied cobinamide, adenine and DMB to synthesize B_{12} and pseudo-B_{12} was assessed according to previously described methods (Anderson *et al.*, 2008). Briefly, a log-phase culture of *S. enterica* was harvested by centrifugation then resuspended in E media (Vogel and Bonner, 1956) that lacked citric acid but possessed ethanolamine (Sigma) as a carbon source hence Non-Citrate Ethanolamine (NCE) media. Cells were inoculated into NCE media (see Appendix B) at an OD_{600} of 0.1 into the following combinations: 20 nM cobinamide alone, 20 nM cobinamide plus 20 nM adenine, 20 nM cobinamide plus 20 nM DMB, 20 nM B_{12} and no supplement. Cultures were incubated at 37°C with shaking and OD_{600} was measured every 2.5 h.

2.12 Phenotypic characterization of mycobacterial mutants

Mycobacterial mutants were primarily characterized by comparing their growth kinetics in liquid media. The inability to complement growth on solid media with selected supplements (e.g. $CoCl_2$) necessitated this. Once optimal precursor concentrations and growth kinetics was established in the fast-growing MSM mutants, this was applied to the panel of MTB.

2.12.1 Characterization of MSM B_{12} mutants

- **Complementation of MSM growth by various**

MSM B_{12} and methionine biosynthetic mutants; Δ*metE*::*hyg*, Δ*cobK*, Δ*cobK* Δ*metE*::*hyg* (Dawes, unpublished) and mc^2155 was assessed for their potential to utilize substrates such as vitamin B_{12} (CNCbl), the principal B_{12} component, cobalt, and L-methionine to complement growth. Briefly, 1 ml of a log-phase culture was harvested by centrifugation at 15000 x g for 10 min, then washed twice in 0.05% $Tween_{80}$ and then resuspended in 7H9/GS liquid media. Two hundred µl of 10-fold serial dilutions were plated on 7H10/GS agar plates containing the desired supplements. After 72 h of incubation at 37°C, CFUs were enumerated. Vitamin B_{12} and L-methionine were utilized at final concentrations of 10 µg/ml (8 µM) and 40 µg/ml (32 µM), respectively. In addition to supplementing growth, a range of cobalt toxicity was established by spotting 10 µl of 10-fold serial dilutions on 7H10/GS agar plates containing either $CoCl_2$ (Sigma-Aldrich) or $CoSO_4$ (Sigma-Aldrich) ranging from 2 µM to 10 mM.

- **Assessment of B_{12} and pseudo-B_{12} synthesis**

The ability of MSM to utilize precursors to synthesize B_{12} and pseudo-B_{12} was assessed by comparing growth kinetics of the MSM B_{12} auxotroph (Δ*cobK* Δ*metE*::*hyg*) supplemented with cobinamide, adenine and DMB. Briefly, 50 ml of log-phase culture grown in 7H9/GS plus B_{12} (10 µg/ml), was harvested by centrifugation at 1100 x g for 10 min then washed twice in 0.05% $Tween_{80}$ and resuspended in Sauton's minimal liquid medium. Thereafter, cells were inoculated at an OD_{600} of 0.1 in 25 ml of Sauton's medium in the following combinations: 10 µM cobinamide; 10µM cobinamide plus 10 µM adenine; 10 µM cobinamide plus 10 µM DMB; 10 µg/ml B_{12} and no supplement. Cultures were incubated at 37°C with shaking. Optical density of the cultures was measured every 24 h, and

cultures were subsequently inoculated into fresh media containing the identical supplement. This process was repeated for up to 72 h. After inoculation at 72 h, the optical density of the cultures was measured every 3 h and recorded.

2.12.2 Characterization of MTB mutants

- **Assessment of B_{12} and pseudo-B_{12} synthesis**

The ability of a panel of MTB mutants to synthesis B_{12} and pseudo-B_{12} from precursors was performed in a similar manner to MSM with a few exceptions. Briefly, a 50 ml log-phase culture grown in 7H9/OADC plus B_{12} (10 µg/ml), where necessary, was harvested by centrifugation at 1100 x g for 10 min, then washed twice in 0.05% $Tween_{80}$ and resuspended in Sauton's medium. Thereafter, cells were inoculated at an OD_{600} of 0.1 in 25 ml of Sauton's in the following combinations: 10 µM cobinamide; 10 µM cobinamide plus 10 µM adenine; 10 µM cobinamide plus 10 µM DMB; 10 µg/ml B_{12} and no supplement. Cultures were incubated flat in tissue culture flasks at 37°C with aeration every second day. Optical density was measured after 8 or 14 days and cultures were then inoculated into exactly the same combinations. Thereafter optical density was measured every second day and recorded.

- **Assessment of putative B_{12} transporter in valerate**

The role of *PPE2* as a putative B_{12} transporter was assessed according to the ability of the mutant to transport B_{12} when grown on valerate in the presence of the isocitrate lyase inhibitor 3-NP, as previously described (Savvi *et al.*, 2008). Briefly, a 50 ml culture (OD_{600} 0.4-0.6) grown in 7H9/OADC plus B_{12} (10 µg/ml) where necessary, was harvested by centrifugation at 1100 x g for 10 min then resuspended in valerate

(Appendix B). Cells were inoculated into 25 ml of media containing valerate plus 3-NP (10 µg/ml), valerate plus 3-NP plus B_{12}, or valerate alone, at an OD_{600} of 0.02. Cultures were incubated in tissues culture flasks at 37°C and optical density was measured every 48 h.

2.13 Semi-quantitative analysis of gene expression

The primers used for semi-quantitative RT-PCR were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are shown in Table 2.8. All primers were designed to amplify DNA fragments that were internal to the open reading frames of the genes of interest.

2.13.1 RNA isolation

RNA isolation was carried out according to a modified protocol by K. Downing *et al.* (2004). Briefly, MTB cultures grown to an OD_{600} of 0.4-0.6 were split into two equal aliquots, B_{12} (10 µg/ml) was added to one and cultures were returned to the incubator. Both cultures were harvested by centrifugation at 1100 x g for 15 min at 0 and 10 h post B_{12} addition. The pellet was resuspended in 2 ml of TRIzol (Sigma, USA), and then transferred to Lysing Matrix B tubes (Qbiogene, USA) and ribolysed three times for 20 s at speed 6 using the Savant Fastprep FP120 with 2 min intervals of cooling on ice between pulses. Samples were centrifuged at 15000 x g for 45 s and supernatants were transferred to Phase Lock gel tubes (Merck) containing 300 µl of chloroform:isoamyl alcohol (24:1). The solution was inverted rapidly for 15 s to mix, then periodically for 2 min thereafter, and centrifuged at 15000 x g for 5 min. The aqueous phase was transferred to microfuge tubes containing an equal volume of

isopropanol and samples were precipitated overnight at 4°C. Nucleic acids were collected by centrifugation at 15000 x g for 20 min, and pellets were washed with 70% ethanol, air-dried and then resuspended in Diethylprocarbonate (DEPC) treated water. Samples were treated with DNase I (Ambion) to eliminate contaminating genomic DNA and then purified with the RNeasy kit (Qiagen) according to the manufacturer's instructions. The samples were further treated with Turbo DNase I (Ambion) according to the manufacturer's instructions. The RNA quality was assessed by electrophoresis on a 2% agarose gel containing 0.1% SDS and quantified using the Nanodrop ND-1000 Spectrophotometer.

2.13.2 Reverse transcription

Reverse transcription reactions were carried out using the Enhanced Avian HS RT-PCR kit (Sigma) as per the manufacturer's instructions. Briefly, primers were annealed to the RNA in a 21 µl reaction consisting of 1 µg of RNA and 0.5 µM of each reverse primer (Table 2.8). The RNA was denatured at 94°C for 90 s, and then annealed at 65°C for 3 min followed by 3 min at 57°C. Thereafter, 10 µl of the annealing mixture was added to the RT reaction which consisted of 1x Avian Myeloblastoma Virus (AMV) RT buffer, 4 mM $MgCl_2$, 200 µM of each dNTP, 0.6 µl of dimethyl sulphoxide (DMSO), and 2U of Enhanced AMV RT (Sigma). The RNA was reverse transcribed at 60°C for 30 min, denatured at 95°C for 5 min and then cooled to 4°C. Control reactions that lacked the RT enzyme were run in parallel to monitor DNA contamination.

Table 2.8: Oligonucleotides used in RT-PCR

Name	Sequence (5'-3')
metE L	TGGTTCGACACCAACTACCA
metE R	GCCCTAACGCCTCTTTGAGT
sigA L	CCTACGCTACGTGGTGGATT
sigA R	CTTGTTGATCACCTCGACCA

2.13.3 PCR

Two µl of the synthesized cDNA (described above) was used as template in a 50 µl PCR reaction that consisted of 1x FastStart PCR buffer without $MgCl_2$ (Roche), 4 mM $MgCl_2$ (Roche), 200 µM of each dNTP (Roche), 5 µl of DMSO (Sigma), 0.5 mg/ml Bovine Serum Albumin (BSA, Roche), 0.2 µM of each primer and 2.5 U of FastStart Taq DNA Polymerase (Roche). The reaction was denatured at 94°C for 10 min, followed by 14 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 30 s and then 24 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 30 s. PCR products were separated on a 4% agarose gel.

3. Results

The genome sequence of MTB H37Rv (Cole *et al.*, 1998) provided a crucial resource to investigate the biology of this formidable pathogen. Among other previously unknown physiological traits, comparative genomic analysis revealed that MTB possesses a near-complete genetic repertoire for the biosynthesis of B_{12} (Rodionov *et al.*, 2003). This was intriguing as vitamin B_{12} biosynthesis represents one of the most complex pathways in nature, and is restricted to certain prokaryotes only. Although consistent with the presence in the genome of three B_{12}-dependent enzymes (Dawes *et al.*, 2003; Warner *et al.*, 2007; Savvi *et al.*, 2008), the inclusion of MTB among the select group of organisms predicted to catalyze *de novo* B_{12} biosynthesis suggested that vitamin B_{12} metabolism might be critical for pathogenesis. Previous studies in the MMRU demonstrated that MTB does not synthesize B_{12} *de novo* (Warner *et al.*, 2007; Savvi *et al.*, 2008); however, the possibility exists that transport of the complete cofactor - or salvage and utilization of vitamin B_{12} precursors from host sources, as well as alternate forms such as pseudo-B_{12} – might assume increased importance during growth or survival under unfavourable conditions encountered through the course of host infection. Therefore, this study aimed to investigate the functionality of the vitamin B_{12} biosynthetic pathway in MTB by testing the ability of a panel of mycobacterial mutants to utilize specific B_{12} precursors and building blocks, including those required for the synthesis of alternate B_{12} forms. In addition, the presence of a B_{12}-responsive regulatory element – a riboswitch – directly upstream of a putative operon containing *PPE2* prompted an investigation of the potential role of the encoded PPE-family protein in B_{12} homeostasis.

3.1 Investigation into mycobacterial B_{12} metabolism utilizing MSM as a surrogate for MTB

3.1.1 Identification of putative homologues of the B_{12} biosynthetic pathway and B_{12}-dependent enzymes in MSM

Previous studies in the MMRU (Warner and Dawes, unpublished) identified putative homologues of B_{12} biosynthetic genes in MTB, which enabled the construction of a proposed B_{12} biosynthetic pathway utilizing a combination of bioinformatics tools (Figure 3.9). In order to establish the appropriateness of MSM as a model for vitamin B_{12} biosynthesis in MTB, this study was initiated by identifying putative MSM homologues of the predicted MTB B_{12} biosynthetic genes. To this end, a BLAST homology search (Altschul *et al.*, 1990) of the MSM database (http://tigerblast.tigr.org/cmr.blast/) was performed using MTB query sequences. This analysis revealed minor differences between the B_{12} biosynthetic gene complements of MSM and MTB. For example, the precorrin-6A synthase encoded by CobF in *P. denitrificans* (Debussche *et al.*, 1993; Min *et al.*, 1993) is absent in MTB and *M. bovis*, but there is a putative homologue in MSM that is encoded by MSMEG_5548. In addition, the aminotransferase encoded by CobC in MTB appears to have no homologue in MSM, though it is possible that a non-orthologous protein fulfils this function. Similar to MTB, the MSM B_{12} biosynthetic pathway contains signatures of aerobic B_{12} biosynthesis including CobG (MSMEG_3871) which requires molecular oxygen for activity, as well as CobK (MSMEG_3875) and CobJ (MSMEG_3873) which contain conserved residues that are associated with aerobic B_{12} synthesis (Shearer *et al.*, 1999). In a parallel analysis of B_{12}-dependent enzymes in the different mycobacteria, it was notable that there appeared to be no homologue of NrdZ in MSM: only MetH and MutAB were found. Furthermore, MSM possesses putative homologues of additional B_{12}-dependent enzymes that

are not present in MTB: specifically, glutamate mutase (MSMEG_0969), dioldehydratase (MSMEG_6318), small and large subunits of ethanolamine ammonium lyase (MSMEG_1553-1554) and three glycerol dehydratases made up of large and small subunits (MSMEG_1547-1548, MSMEG_6320-6321 and MSMEG_0496-0497). Therefore, while MSM does not possess the same vitamin B_{12}-dependent complement that characterizes the MTB genome, this non-pathogenic mycobacterium appears to encode the full machinery required to synthesize B_{12} *de novo*.

Based on these genetic similarities, MSM was initially used as a surrogate (1) to investigate the ability of vitamin B_{12} (CNCbl) and L-methionine to complement growth of mutants containing disruptions in B_{12} and methionine biosynthesis pathways; and (2) to assess the ability of MSM to utilize vitamin B_{12} precursors and building blocks including cobinamide, DMB, and adenine to synthesize different forms of B_{12}, as well as to optimize the precursor concentrations and experimental conditions before proceeding into MTB.

3.1.2 Complementation of MSM mutants with vitamin B_{12} and L-methionine

Mutants of the enteric bacteria, *S. typhimurium* and *E. coli*, which contain disruptions in their B_{12}-independent methionine synthases, encoded by *metE* in both cases, are unable to grow unless supplemented with either L-methionine or vitamin B_{12} (Davis and Mingioli, 1950; Jeter *et al.*, 1984). This methionine – and, indirectly, vitamin B_{12} – auxotrophy established that the alternative, vitamin B_{12}-dependent methionine synthase, MetH, was not able to complement loss of the B_{12}-independent enzyme in these organisms. In turn, it also suggested that *S. typhimurium* and *E. coli* could not synthesize B_{12} *de novo* under standard laboratory conditions. This was

subsequently verified with the demonstration that both organisms synthesize B_{12} under anaerobic conditions only; however, it established an assay – chemical complementation with methionine or vitamin B_{12} – that could be usefully applied to other organisms including MTB (Warner *et al.*, 2007; Savvi *et al.*, 2008).

To investigate the ability of MSM to synthesize vitamin B_{12}, a panel of B_{12} and methionine biosynthetic mutants was constructed (Dawes, unpublished) and their ability to utilize B_{12} and L-methionine was assessed in a range of *in vitro* assays. As expected, a Δ*cobK* mutant grew in the absence of B_{12} supplementation (Figure 3.1), owing to the function of the B_{12}-independent methionine synthase, MetE. Unlike the corresponding MTB mutant (Warner *et al.*, 2007), the MSM *metE* knockout was able to grow with similar kinetics to the parental mc^2155 (Figure 3.1). This was surprising and suggested that, in contrast to MTB (as well as *S. typhimurium* and *E. coli*), MSM is capable of *de novo* B_{12} biosynthesis *in vitro* under standard (aerobic) culture conditions. Confirming this interpretation, the double Δ*cobK* Δ*metE*::*hyg* mutant was unable to grow in the absence of exogenous vitamin B_{12}. As a strict B_{12} auxotroph, the MSM Δ*cobK* Δ*metE*::*hyg* double mutant therefore phenocopies the MTB Δ*metE*::*hyg* mutant (Warner *et al.*, 2007). An interesting feature of the MTB Δ*metE*::*hyg* mutant is that it cannot be supplemented with L-methionine on solid 7H10 agar (Warner, 2006). In another departure from the MTB phenotype, the MSM Δ*cobK* Δ*metE*::*hyg* double mutant was able to utilize exogenous methionine for growth *in vitro* on 7H10 agar (Figure 3.1). However, the colonies of the Δ*metE*::*hyg* and Δ*cobK* Δ*metE*::*hyg* mutants appeared significantly smaller in size compared to the wild–type strain, which suggested that methionine could only partially restore growth in strains lacking *metE*.

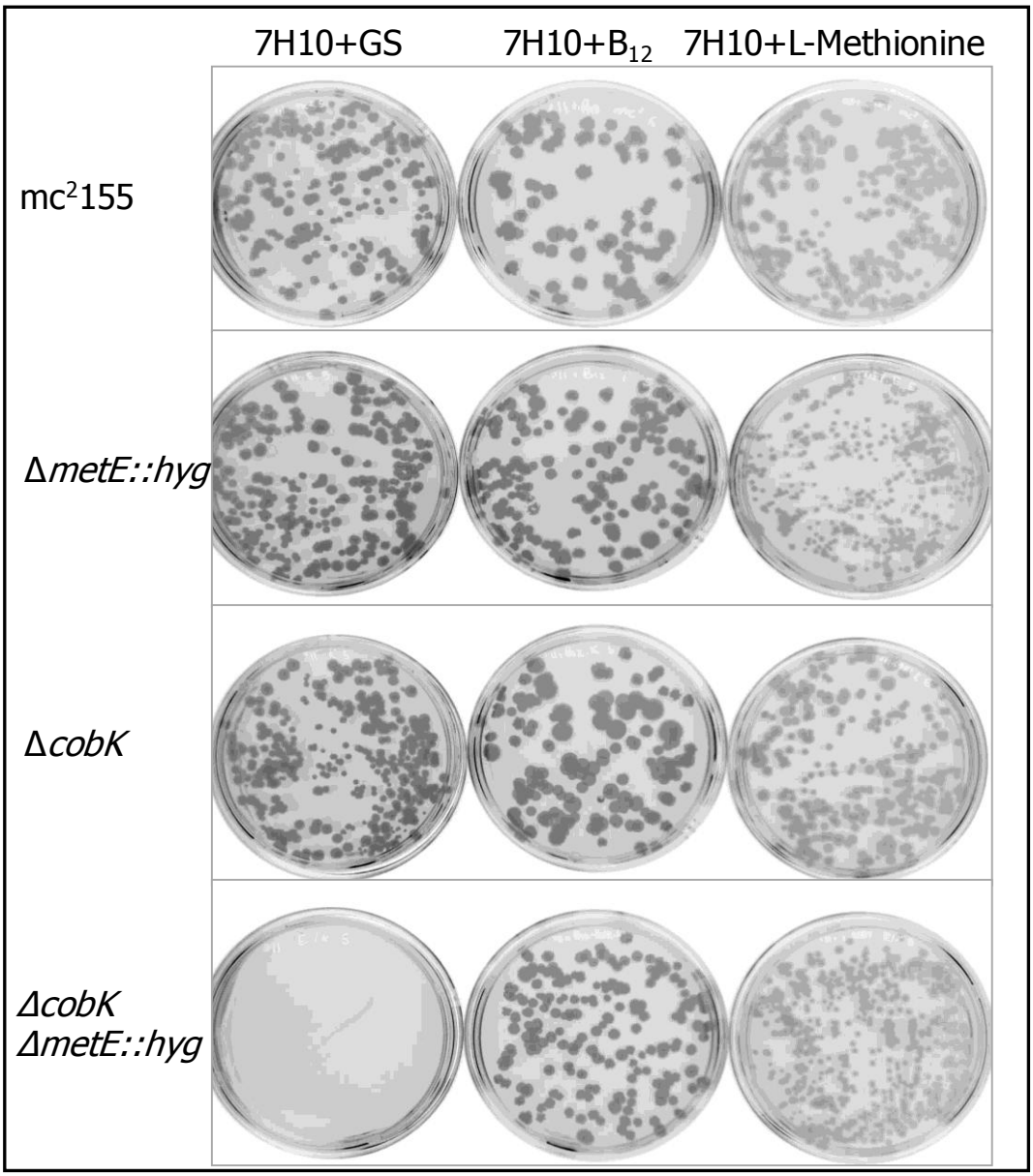

Figure 3.1: Growth of MSM B_{12} and methionine biosynthetic mutants. A 200 µl aliquot of a 10-fold serial dilution of a log phase culture of each strain was plated on 7H10+GS, 7H10+B_{12} (10 µg/ml), and 7H10+L-methionine (40 µg/ml) and growth was scored after 72 h. Growth of Δ*metE*::*hyg* and Δ*cobK* mutants was comparable to mc^2155 on all substrates, whereas the Δ*cobK* Δ*metE*::*hyg* double mutant was unable to grow without B_{12} or L-methionine supplementation.

3.2 Can MSM and MTB synthesize alternate forms of the B_{12} cofactor?

3.2.1 Synthesis of B_{12} and pseudo-B_{12} by mycobacterial B_{12} auxotrophs

Subsequent to the initiation of this project, Anderson *et al.* (2008) demonstrated the ability of *S. enterica* to synthesize and utilize an alternate form of B_{12} known as pseudo-B_{12}. The key difference between B_{12} and pseudo-B_{12} lies in the α-axial ligand: in canonical ("orthodox") vitamin B_{12}, this position is occupied by DMB. Substituting DMB with the purine nucleobase, adenine, generates pseudo-B_{12}. Under strict anaerobic conditions, *S. enterica* is able to synthesize either form - DMB-containing "orthodox" B_{12} or pseudo-B_{12}. However, during aerobic growth, it must be provided with cobinamide (the corrin ring plus aminopropanol side chain) and DMB in order to synthesize B_{12}. Notably, Anderson and colleagues also showed that the synthesis of pseudo-B_{12} requires the same set of enzymes (CobT, CobU, CobS and CobC) that are involved in the synthesis of DMB-containing B_{12} (Anderson *et al.*, 2008). Their results raised the possibility that mycobacteria might possess a similar ability to synthesize either B_{12} or pseudo-B_{12}, depending on whether bacilli are provided with cobinamide plus DMB (for orthodox B_{12}) or cobinamide plus adenine (pseudo-B_{12}). If so, this would imply that pseudo- B_{12} could act as a functional equivalent of B_{12} in B_{12}-dependent pathways, thereby enabling MTB to utilize alternate B_{12} forms depending on prevailing environmental (host) conditions.

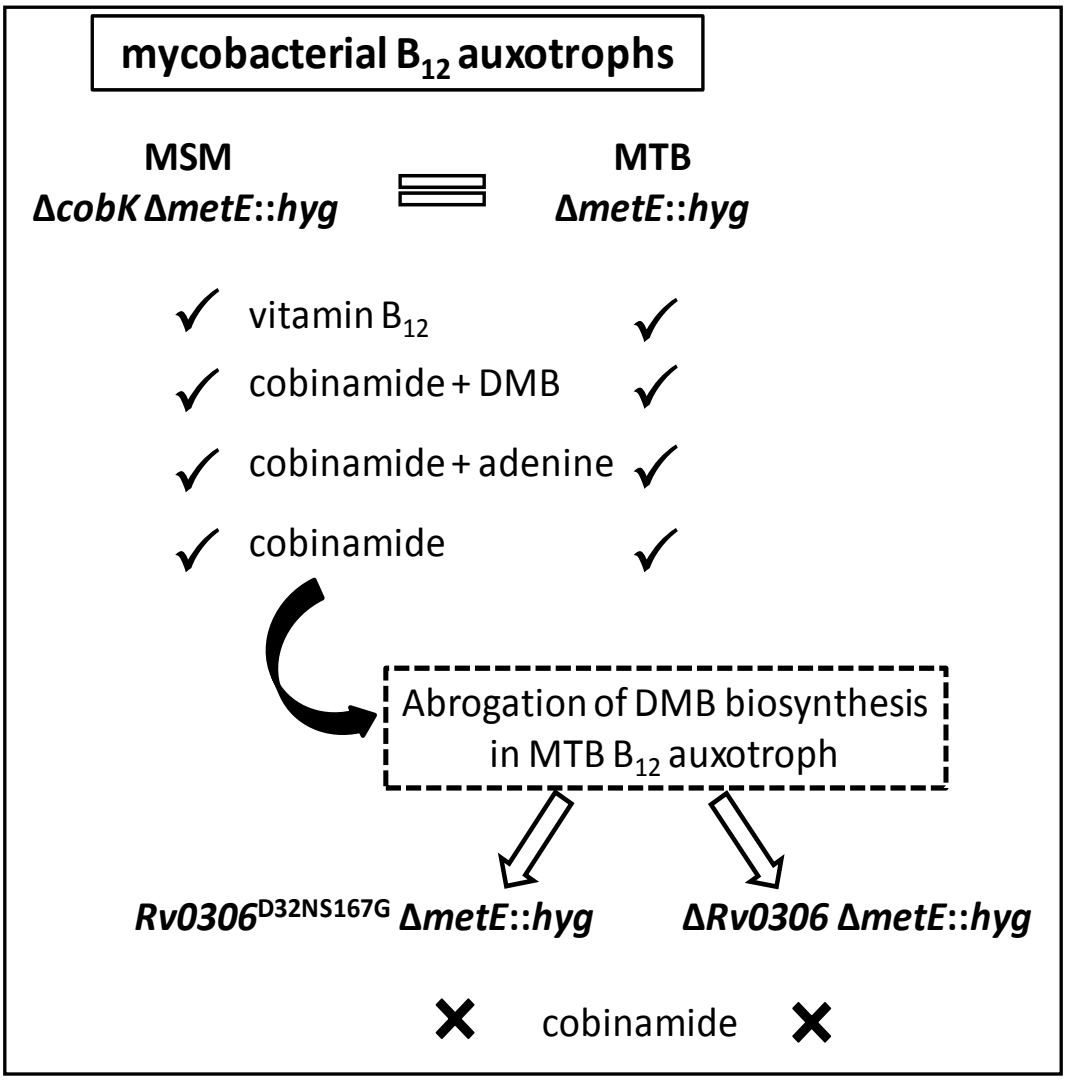

Figure 3.2: Schematic illustrating predicted phenotypes in assessment of pseudo-B_{12} synthesis in mycobacteria. MSM Δ*cobK* Δ*metE*::*hyg* and MTB Δ*metE*::*hyg* both require B_{12} for growth. Both strains are predicted to grow when supplemented with 'orthodox' B_{12} precursors (cobinamide plus DMB), pseudo-B_{12} precursors (cobinamide plus adenine) and cobinamide. However, growth on cobinamide alone is indicative of endogenous DMB synthesis. Thus, it is predicated that abrogation of DMB synthesis in MTB will result in the inability of *Rv0306*^{D32NS167G} Δ*metE*::*hyg* and Δ*Rv0306* Δ*metE*::*hyg* to utilize cobinamide alone. Symbols: **=** equivalent; ✓ growth; **✕** no growth.

3.2.2 Aerobic growth of S. enterica *supplemented with B_{12} and pseudo-B_{12} precursors*

Before testing the hypothesis that MTB might be able to utilize alternate B_{12} forms, the ability to reproduce the experimental protocol developed for *S. enterica* by Anderson *et al.* (2008) was validated. To this end, the growth kinetics of *S. enterica* were assessed using a variation of Vogel and Bonner's (1955) E media. In their study, Anderson *et al.* (2008) referred to this medium as Non-Citrate E (NCE) media, as it is a minimal medium that is deficient in citrate but contains ethanolamine as carbon source. *S. enterica* utilizes ethanolamine as the sole carbon and nitrogen source under aerobic conditions only if B_{12} or, in this case, B_{12} precursors are provided (Chang and Chang, 1975). After growth to log phase in LB broth, cultures were harvested and washed before inoculating into NCE medium at an OD_{600} of 0.1. Growth was recorded every 2.5 h. As indicated in Figure 3.3A, *S. enterica* was able to utilize cobinamide plus DMB to synthesize B_{12} under aerobic conditions; cobinamide alone was not sufficient for growth. Moreover, an adenine titration was performed which established that the growth of *S. enterica* improved with increasing concentrations of adenine (Figure 3.3B). These data were consistent with the results of Anderson *et al.* (2008), and confirmed that the cobinamide, DMB and adenine supplements provided were active and capable of supporting bacterial growth.

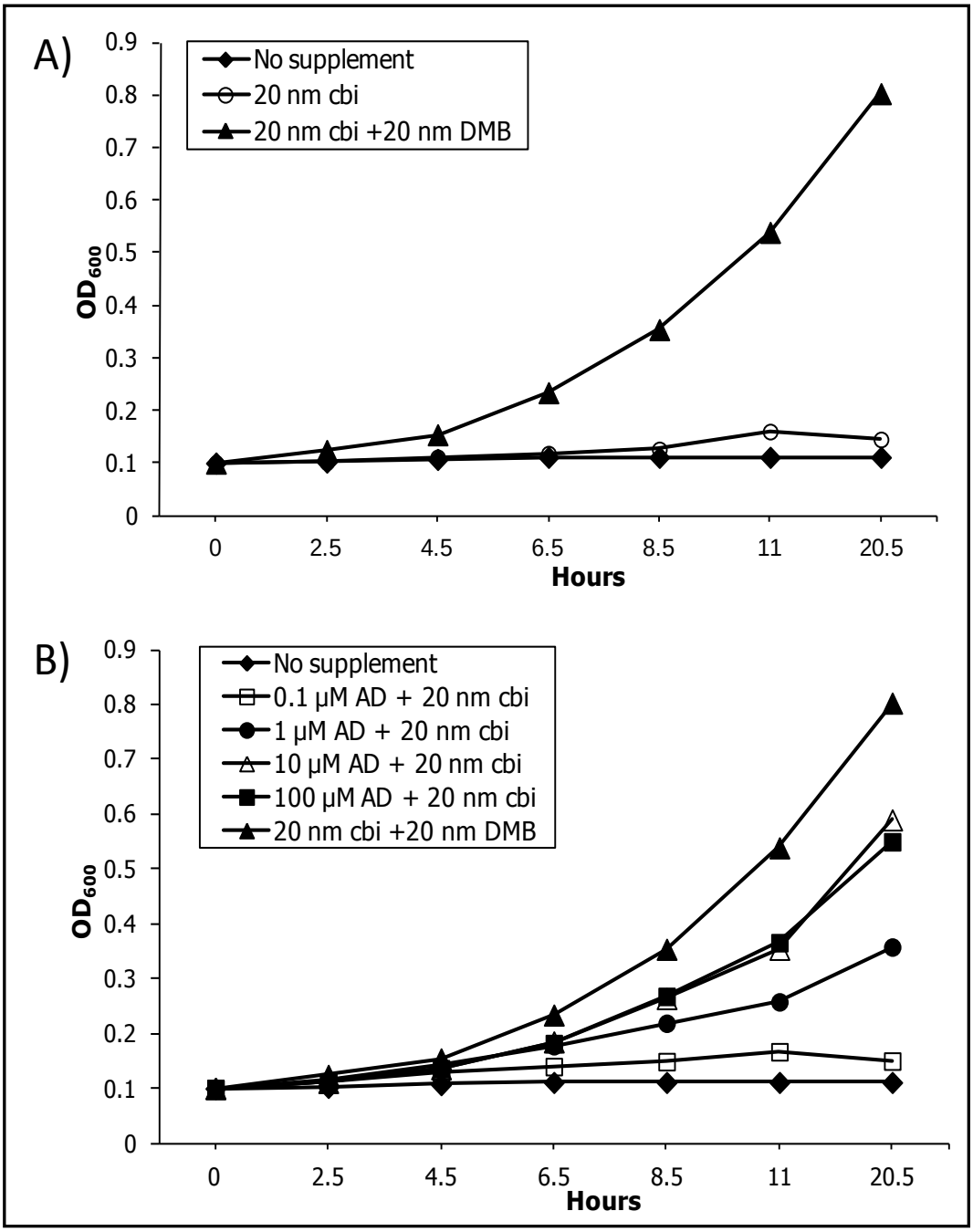

Figure 3.3: Aerobic synthesis of B_{12} and pseudo-B_{12} by *S. enterica.* *S. enterica* was grown in NCE minimal medium with ethanolamine as the sole carbon and nitrogen source (A) without any supplement (◆), with 20 nM cobinamide (○), and with 20 nM cobinamide plus 20 nM DMB (▲), and (B) with a fixed concentration of 20 nM cobinamide plus increasing concentrations of 0.1 µM adenine (□), 1 µM adenine (▲), 10 µM adenine (△), 100 µM adenine (■) and 20 nM DMB (●). Abbreviations: cbi - cobinamide, AD - adenine.

3.2.3 MSM utilizes B_{12} and pseudo-B_{12} precursors

In addition to assessing the ability of MSM to utilize B_{12} and pseudo-B_{12} precursors, this faster growing mycobacterium was used to determine the optimal concentrations of pseudo-B_{12} (cobinamide and adenine) and B_{12} (cobinamide and DMB) precursors for use in MTB. As noted in Figure 3.9, comparative bioinfomatic analyses identified a near-complete B_{12} biosynthetic pathway in MSM, including homologues of enzymes required for DMB - and, by inference, adenine - attachment (CobT, CobU, CobS). Rv2228c and MSMEG_4305 are the putative homologues of *S. enterica* CobC in MTB and MSM, respectively. The gene annotated as *cobC* in MTB is homologous to the aminotransferase encoded by *cobD* in *S. enterica*. As MSM synthesizes B_{12} *in vitro* (Figure 3.1), the Δ*cobK* Δ*metE*::*hyg* double mutant containing disruptions in both B_{12}-independent methionine synthase (*metE*) and Cbl biosynthesis (*cobK*) genes were used as the experimental strain. Deletion of *cobK* was predicted not to impact the ability of MSM to utilize the supplied cobinamide and DMB supplements for B_{12} (or pseudo-B_{12}) synthesis since this strain retains all components of the late-stage B_{12} biosynthetic pathway (Figure 3.9). This includes CobT which possesses a broad specificity (Cheong *et al.*, 2001) and can phosphoribosylate a wide variety of aromatic substrates giving rise to a variety of lower B_{12} ligands. When *S. enterica* is grown under aerobic conditions, DMB occupies the β-ligand (Johnson and Escalante-Semerena, 1992) whereas, under anaerobic conditions, it is replaced by adenine (Keck and Renz, 2000). In both cases, this transfer is carried out by CobT. Therefore, CobT should facilitate the assimilation of DMB or adenine into the α-ribazole moiety of adenosyl-cobinamide-GMP (utilizing exogenous supplied cobinamide) (Taga *et al.*, 2007).

As a strict auxotroph, the Δ*cobK* Δ*metE*::*hyg* mutant could be propagated only when grown in media supplemented with vitamin B_{12} (Figure 3.1 & 3.4A). Therefore, for growth assays in liquid media, cells were harvested during log-phase growth in B_{12}-supplemented medium before washing them to remove any residual B_{12}. Thereafter, the cells were re-inoculated at a starting OD_{600} of approximately 0.1 in fresh Sauton's minimal medium. However, for the first generation, this proved insufficient: growth was observed in all media even in the absence of supplement (data not shown). This was presumably because cells retained sufficient quantities of residual (intracellular) B_{12}. Therefore, a modified protocol was adopted for all subsequent experiments in which all cultures were serially re-inoculated into fresh Sauton's medium containing the identical supplement combinations; that is, a culture grown in Sauton's plus B_{12} was serially re-inoculated into fresh Sauton's medium containing B_{12}, another culture grown in Sauton's only was serially re-inoculated into fresh Sauton's medium lacking any supplement, *etc*. This process was repeated every 24 h for a duration of 72 h until all residual (internal) B_{12} was utilized, as determined by the inability of the Δ*cobK* Δ*metE*::*hyg* mutant to grow in supplement-free medium (Figure 3.4A). It should be noted that, in all MSM growth curves, only three time points are shown for samples grown in medium containing B_{12} (Figures 3.4A&B and Figures 3.5A&B). This is because the B_{12}-supplemented samples were characterized by robust growth whereas those strains supplemented with other compounds grew with much slower kinetics. Therefore, for easier visualization of the data, the optical density readings for the B_{12}-supplemented samples were excluded from the growth curves after the third time point.

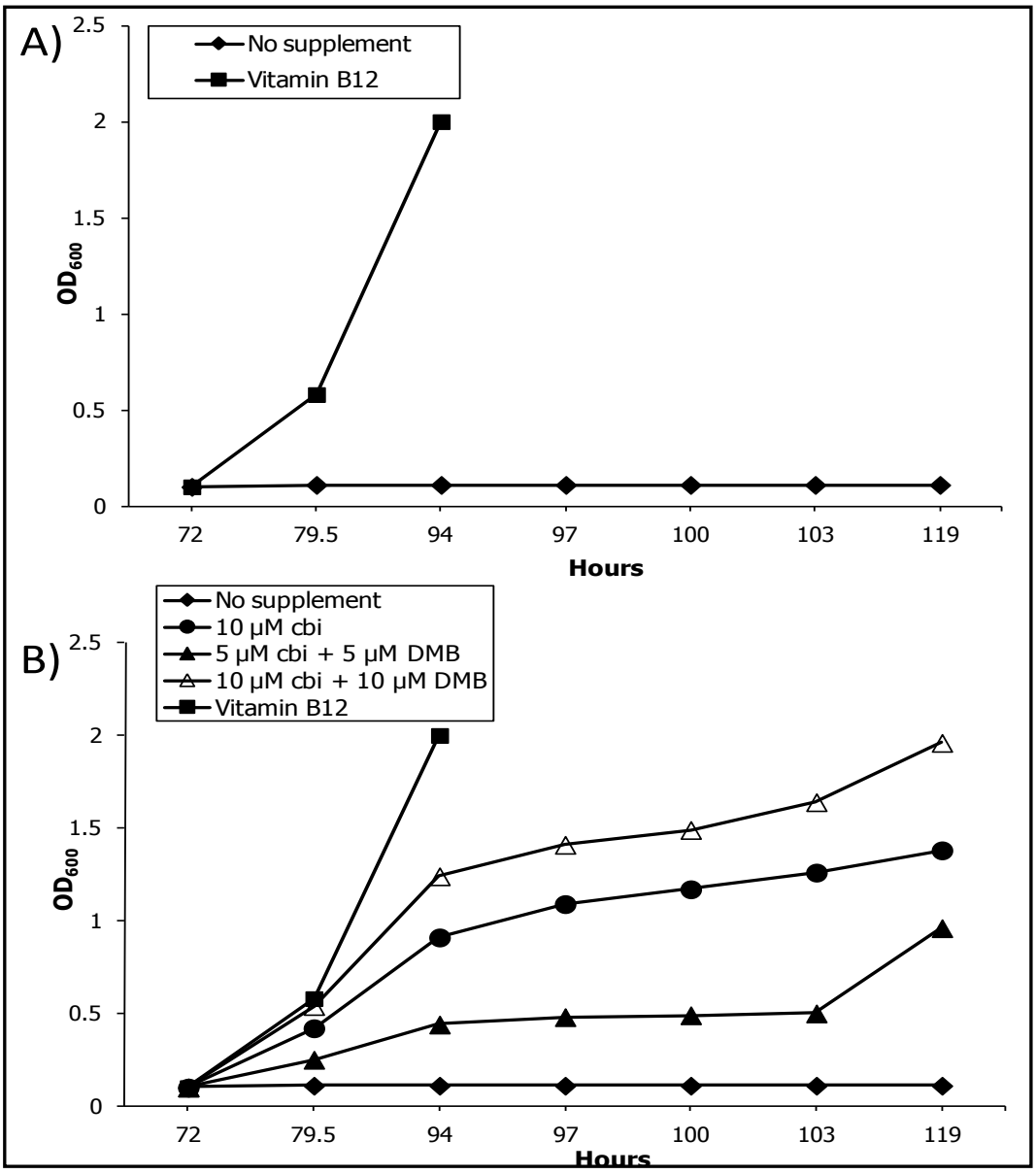

Figure 3.4: Growth kinetics of MSM Δ*cobK* Δ*metE*::*hyg* mutant when supplemented with B_{12} and B_{12} precursors. A log phase culture of Δ*cobK* Δ*metE*::*hyg* was washed before being inoculated at an OD_{600} of 0.1 into Sauton's minimal medium, and then serially re-inoculated into the same medium to deplete internal B_{12} levels. Optical densities were measured every 3 h for approximately 5 days. A) The Δ*cobK* Δ*metE*::*hyg* mutant is a B_{12} auxotroph that requires exogenous B_{12} (10 µg/ml) (■) for growth, and is unable to grow in media lacking B_{12} (◆). B) Growth of the Δ*cobK* Δ*metE*::*hyg* mutant in medium supplemented with cobinamide only (●), with lower concentrations of cobinamide and DMB (▲), with higher concentrations of cobinamide and DMB (△), and with vitamin B_{12} (■) or no supplement (◆). Data are representative of a single experiment from two independent biological replicates. OD_{600} readings of B_{12} supplemented media were curtailed at 94 h. Abbreviations: cbi- cobinamide, AD- adenine.

Importantly, the Δ*cobK* Δ*metE*::*hyg* mutant was able to grow when supplemented with B_{12} precursors (cobinamide plus DMB), strongly suggesting the ability of MSM to assimilate these into vitamin B_{12} (Figure 3.4B). Similar growth was observed when the mutant was supplemented with the pseudo-B_{12} precursors, cobinamide plus adenine (Figure 3.5A). Although this hinted at the possible synthesis (and utilization) of pseudo-B_{12} by MSM, this observation was not conclusive since the mutant was able to grow on cobinamide alone, perhaps indicating the endogenous production and attachment of DMB to the supplemented cobinamide to produce "orthodox" vitamin B_{12}. That is, because the late stage of the B_{12} biosynthetic pathway is retained in the Δ*cobK* Δ*metE*::*hyg* mutant, it was not possible to determine unequivocally whether cobinamide plus adenine, or cobinamide alone, was utilized as the active cofactor. To resolve that question, an adenine titration was performed utilizing increasing amounts of adenine in combination with a fixed concentration of cobinamide, as described by Anderson *et al.* (2008). Increasing concentrations of adenine appeared to correlate with improved growth of the Δ*cobK* Δ*metE*::*hyg* double mutant (Figure 3.5B). Although suggestive of pseudo-B_{12} synthesis, this result was not sufficiently conclusive since the stepwise trend was not perfect and, as observed above, cobinamide alone was associated with good growth. Moreover, the exact nature of the individual chemical species being synthesized cannot be determined utilizing our genetic approach. Instead, a more precise method like high-performance liquid chromatography (HPLC) is required to determine the specific cofactor form, and thus establish whether MSM can indeed synthesize and utilize pseudo-B_{12} as a functional equivalent of B_{12} in key B_{12}-dependent metabolic pathways.

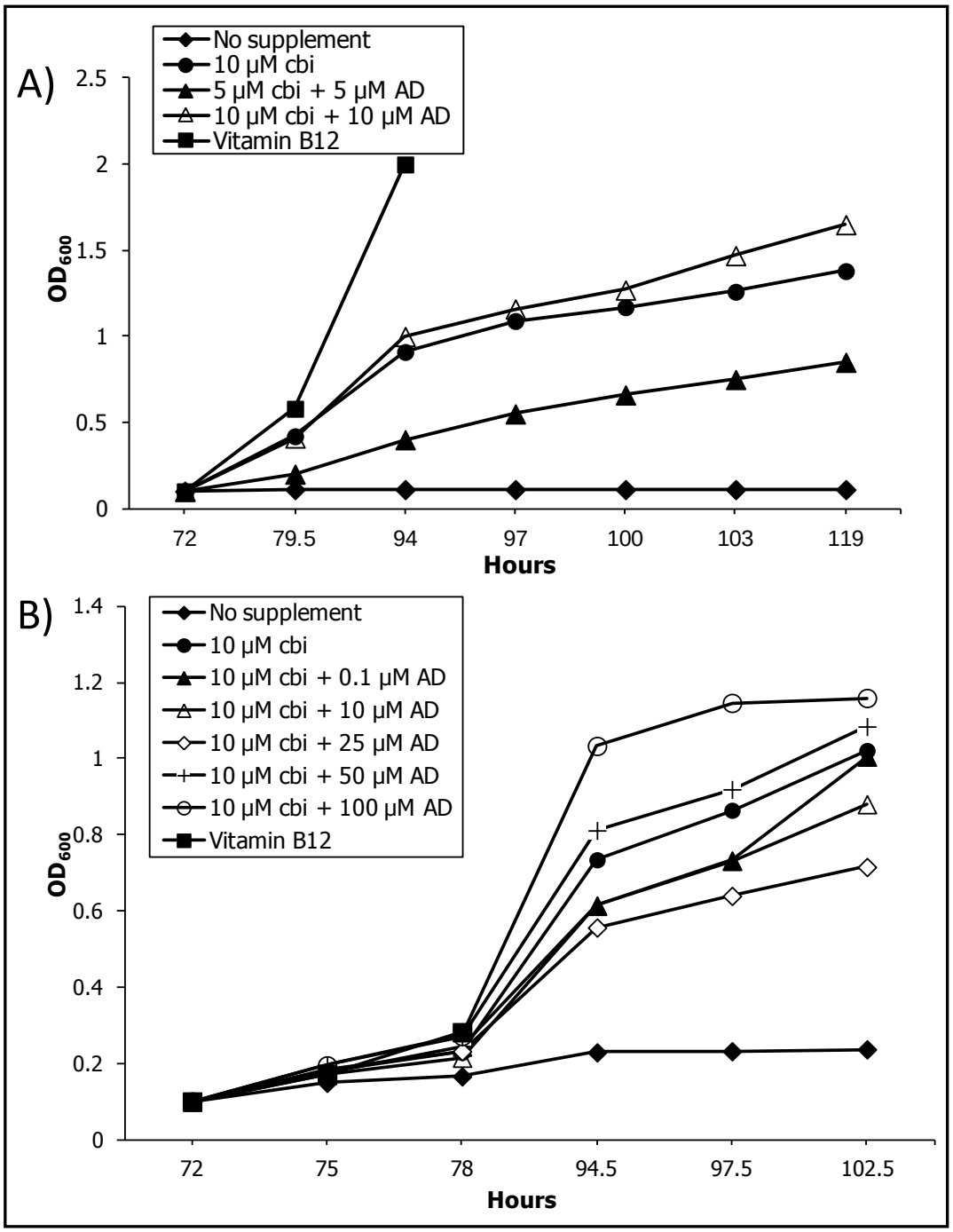
A)
No supplement
10 µM cbi
5 µM cbi + 5 µM AD
10 µM cbi + 10 µM AD
Vitamin B12
OD600
Hours
B)
No supplement
10 µM cbi
10 µM cbi + 0.1 µM AD
10 µM cbi + 10 µM AD
10 µM cbi + 25 µM AD
10 µM cbi + 50 µM AD
10 µM cbi + 100 µM AD
Vitamin B12
OD600
Hours

Figure 3.5: Growth kinetics of MSM Δ*cobK* Δ*metE*::*hyg* when supplemented with B_{12} and pseudo-B_{12} precursors. A log phase culture of Δ*cobK* Δ*metE*::*hyg* was washed before being inoculated at an OD_{600} of 0.1 into Sauton's minimal medium, and then serially re-inoculated into the same medium to deplete internal B_{12} levels. Optical densities were measured every 3 h for approximately 5 days. A) Growth with cobinamide only (●), with lower concentration cobinamide plus adenine (▲), with higher concentration cobinamide plus adenine (△), and with vitamin B_{12} (■) versus no supplement (◆). B) Differential response to increasing concentrations of adenine: 0.1 µM adenine (▲), 1 µM adenine (□), 10 µM adenine (△), 25 µM adenine (◇), 50 µM adenine (+), 100 µM adenine (○), plus fixed concentration of cobinamide at 10 µM (●), no supplement (◆), and 10 µg/ml vitamin B_{12} (■). Data are representative of a single experiment from two independent biological replicates. OD_{600} readings of B_{12} supplemented media were curtailed at 94 h (A) and 78 h (B). Abbreviations: cbi- cobinamide, AD- adenine.

3.2.4 Synthesis of B_{12} and pseudo-B_{12} in MTB

Having established effective B_{12} precursor concentrations in MSM, these were applied to the MTB Δ*metE*::*hyg* mutant (Warner *et al.*, 2007). Analogous to the MSM Δ*cobK* Δ*metE*::*hyg* double knockout, MTB Δ*metE*::*hyg* can only be propagated in media containing B_{12}. Therefore, cells were first washed before inoculating at an OD_{600} of 0.1 into Sauton's minimal medium. Cultures were grown for 8 to 14 days to allow depletion of internal B_{12}, and then re-inoculated into fresh Sauton's medium containing the same supplement(s). The Δ*metE*::*hyg* mutant was able to grow in medium supplemented with cobinamide alone, as well as cobinamide plus DMB (Figure 3.6A). When supplemented with pseudo-B_{12} precursors (cobinamide plus adenine) the MTB B_{12} auxotroph was also able to grow, as shown in Figure 3.6B.

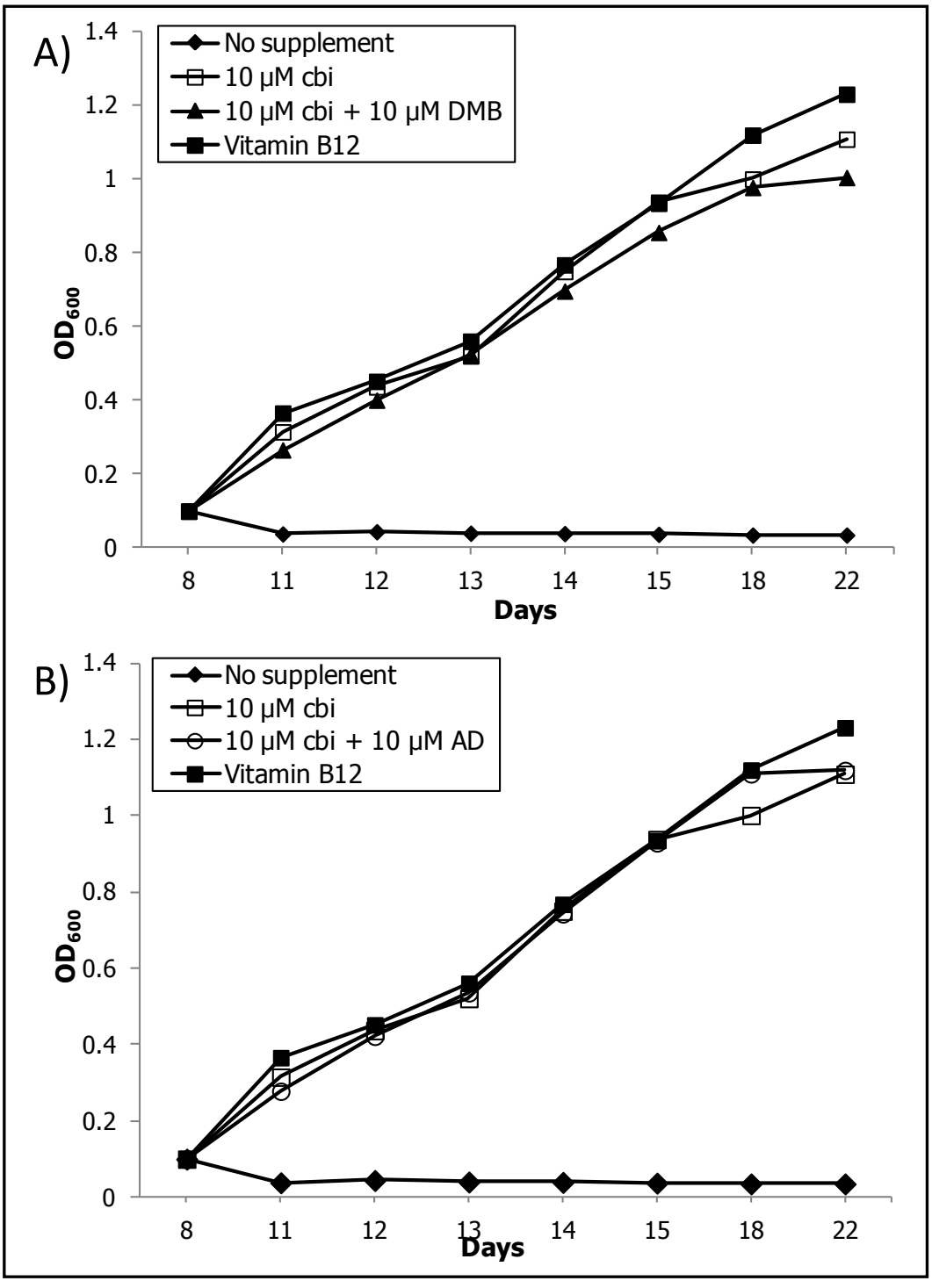
A)
No supplement
10 µM cbi
10 µM cbi + 10 µM DMB
Vitamin B12
OD600
Days
B)
No supplement
10 µM cbi
10 µM cbi + 10 µM AD
Vitamin B12
OD600
Days

Figure 3.6: Growth kinetics of MTB Δ*metE*::*hyg* mutant when supplemented with B_{12} and pseudo-B_{12} precursors. A log-phase culture of MTB Δ*metE*::*hyg* was washed then inoculated at an OD_{600} of 0.1 into Sauton's medium containing the following: no supplement (◆), cobinamide only (□), 10 μM cobinamide plus 10 μM DMB (▲), 10 μM cobinamide plus 10 μM adenine (○) and vitamin B_{12} (■). Cultures were grown for 8-14 days to allow internal B_{12} to be depleted before re-inoculating into fresh Sauton's containing the same supplement(s). (A) Growth with B_{12} precursors, cobinamide plus DMB and (B) growth with pseudo-B_{12} precursors, cobinamide plus adenine. Data are representative of a single experiment from three independent biological replicates. Abbreviations: cbi- cobinamide, AD- adenine.

The ability of B_{12} and pseudo-B_{12} precursors to support the function of other B_{12}-dependent enzymes which require different forms of the cofactor for activity was also investigated. MetH utilizes MeCbl while both MutAB and NrdZ require AdoCbl. To determine the ability of pseudo-B_{12} to support AdoCbl-dependent growth, an assay was employed that was developed previously in the MMRU (Savvi *et al.*, 2008), and involves the use of the succinate analogue, 3-NP, to inhibit ICL function. Among various alternative odd-chain fatty acids, the catabolism of the five carbon (C_5) compound, valerate, as an alternative carbon source generates an equimolar ratio of acetyl-CoA and propionyl-CoA subunits. Propionyl-CoA is metabolized through the methylcitrate and glyoxylate pathways, and its accumulation is toxic to MTB. Wild-type MTB H37Rv is unable to grow on valerate as a sole carbon source in the presence of 3-NP due to toxic build-up of propionyl-CoA (Figure 3.7). However, Savvi *et al.* (2008) demonstrated that 3-NP-mediated growth inhibition is alleviated by the addition of B_{12} which allows the propionyl-CoA to be metabolized through the MutAB-containing methylmalonyl pathway. Supplementation of the medium with various B_{12} precursors resulted in growth of H37Rv grown in valerate plus 3-NP (Figure 3.7). This suggested that MutAB was functional in these conditions, thereby reinforcing the conclusion that MTB can utilize

exogenous B_{12} or cobinamide to support the function of both AdoCbl-dependent (MutAB) and MeCbl-dependent (MetH) pathways.

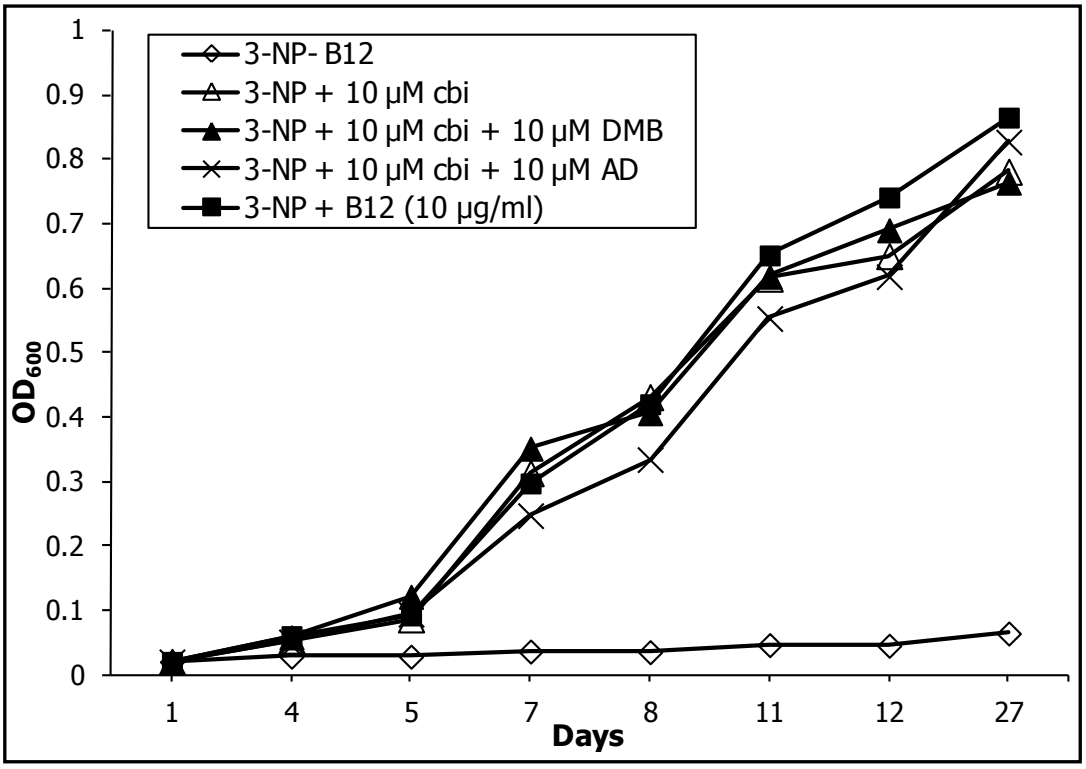

Figure 3.7: Growth kinetics of MTB H37Rv in valerate plus the ICL inhibitor, 3-NP, and supplemented with B_{12} and pseudo-B_{12} precursors. H37Rv was grown in medium containing valerate as the sole carbon source in the presence of 3-NP with vitamin B_{12} (■), without vitamin B_{12} (◇), with 10 µM cobinamide (△), with 10 µM cobinamide plus 10 µM DMB (▲), and with 10 µM cobinamide plus 10 µM adenine (X). Data are representative of a single experiment from three independent biological replicates. Abbreviations: cbi- cobinamide, AD- adenine.

As with MSM, growth of MTB Δ*metE*::*hyg* with cobinamide plus adenine could not be attributed to pseudo-B_{12} biosynthesis since this mutant was also able to grow in media containing cobinamide alone (Figure 3.6A). Again, this suggested the possibility of endogenous DMB biosynthesis. To assess whether increasing concentrations of adenine would influence growth, titrations with fixed concentrations of cobinamide (5 μM or 10 μM) were performed. Unlike MSM (Figure 3.5), no differential growth was seen in MTB in response to increasing concentrations of adenine with 10 μM cobinamide (Figure 3.8B). However, decreasing the cobinamide concentration to 5 μM appeared to correlate with improved growth of Δ*metE*::*hyg* in the presence of elevated adenine concentrations (Figure 3.8A). These data also suggest that the higher concentration of cobinamide (10 μM) might exceed the requirements for growth, and so might conceal the benefits to the bacillus of increasing adenine concentrations in this assay.

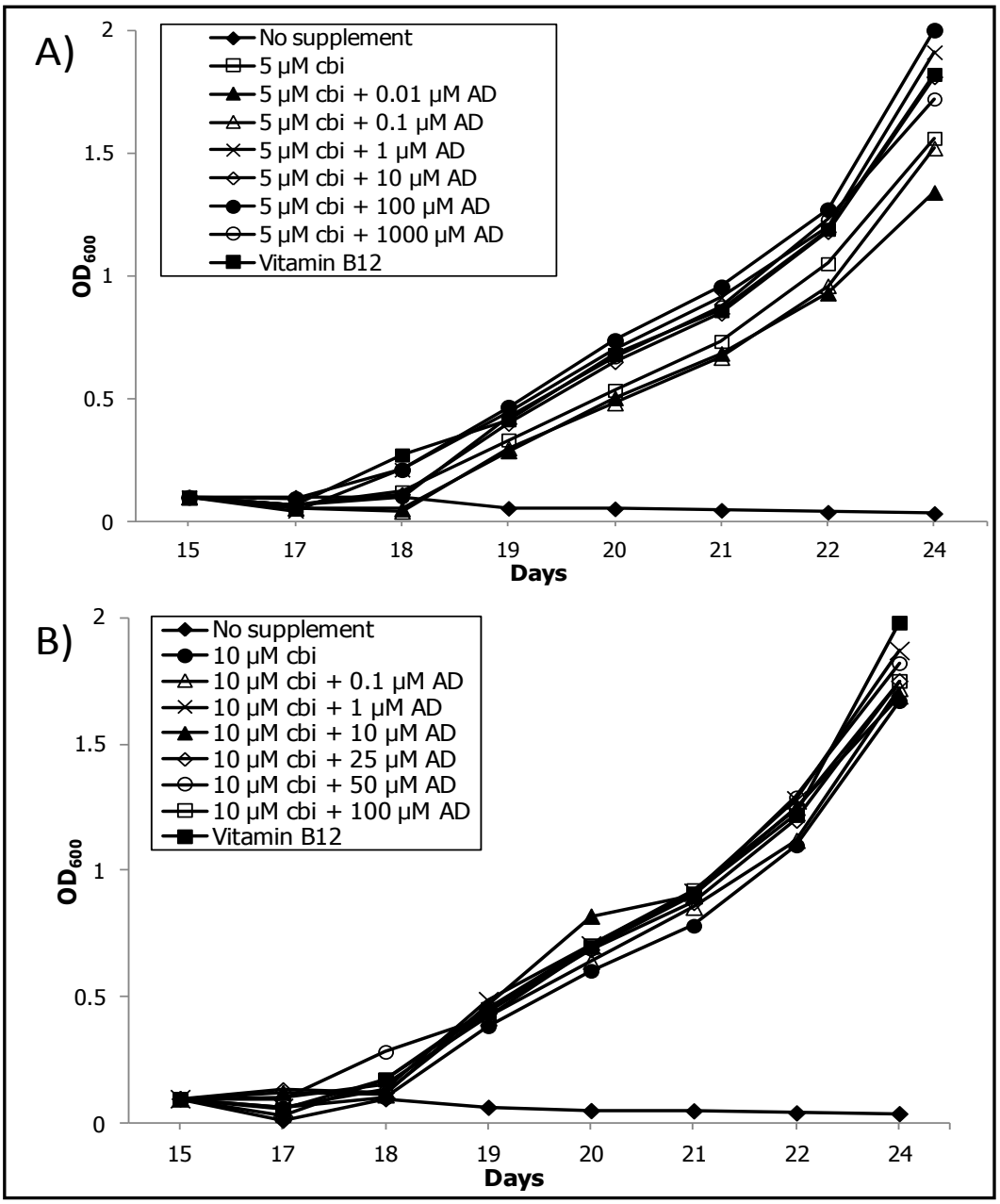

Figure 3.8: Assessment of pseudo-B_{12} synthesis by MTB Δ*metE*::*hyg* by adenine titration. A log-phase culture of MTB Δ*metE*::*hyg* was washed then inoculated at an OD_{600} of 0.1 into Sauton's minimal media, grown for 8-14 days until internal B_{12} was depleted, and then re-inoculated into media containing the exactly same supplement(s). Titrations were performed with fixed concentrations of either (A) 5 µM cobinamide or (B) 10 µM cobinamide, plus increasing concentrations of adenine. Data are representative of a single experiment from three independent biological replicates Abbreviations: cbi-cobinamide, AD-adenine.

3.2.5 Investigation of pseudo-B_{12} synthesis in MTB by abrogation of DMB biosynthesis

To determine whether MTB was able to utilize exogenous DMB (or adenine) for the synthesis of B_{12} (or pseudo-B_{12}), a genetic approach was adopted that entailed the abrogation of DMB biosynthesis. The rationale for this approach was that, by eliminating the ability of Δ*metE*::*hyg* to synthesize its own DMB, the mutant could be "forced" to utilize exogenous B_{12} (cobinamide plus DMB) or pseudo-B_{12} (cobinamide plus adenine) precursors. To date, no biosynthetic genes have been identified in MTB for the synthesis of the lower ligand, DMB. However, recent studies have implicated BluB in the synthesis of DMB in *S. meliloti* (Campbell *et al.,* 2006; Taga *et al.,* 2007).

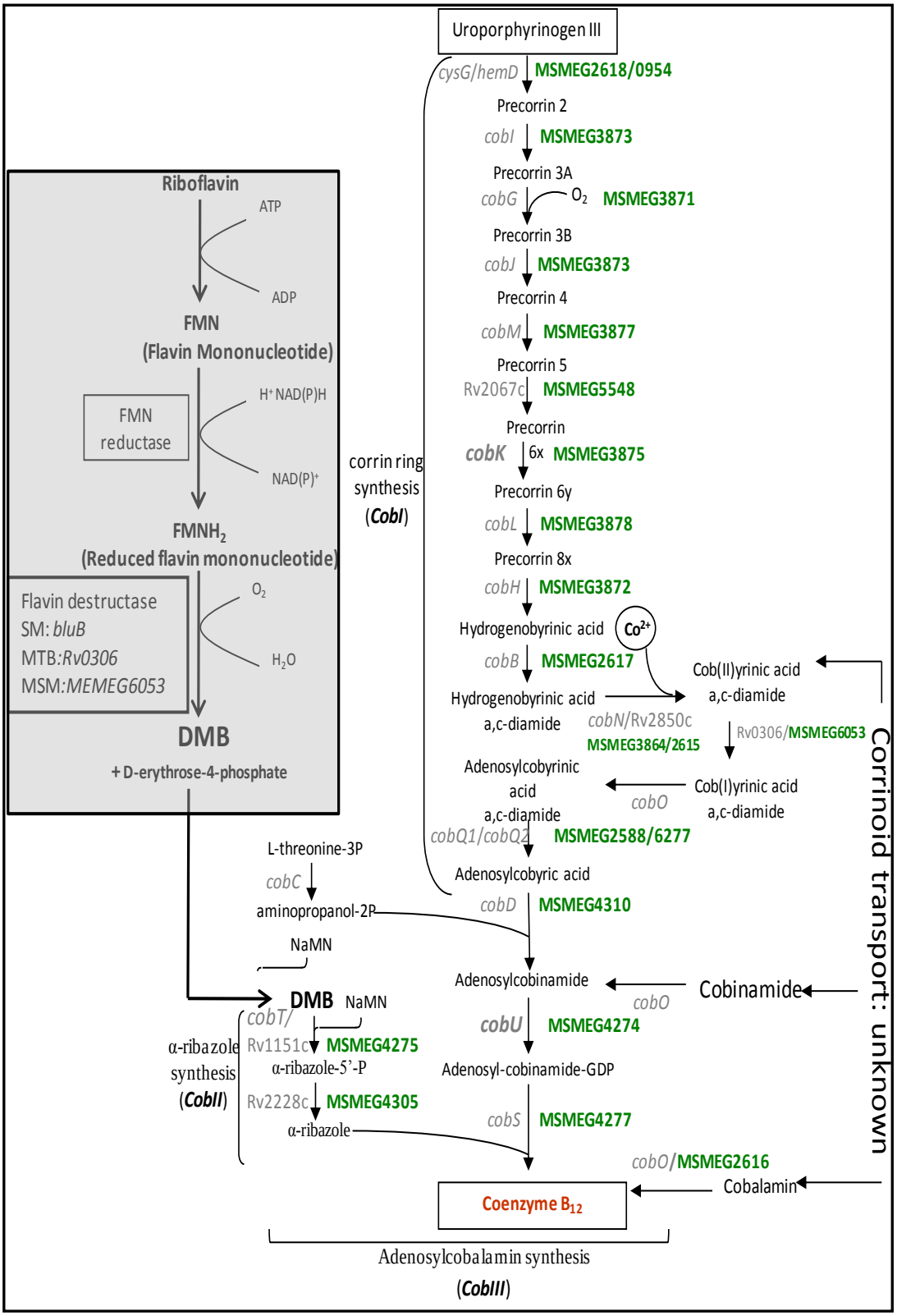
Uroporphyrinogen III
cysG/hemD MSMEG2618/0954
Precorrin 2
cobI MSMEG3873
Precorrin 3A
cobG O2 MSMEG3871
Precorrin 3B
cobJ MSMEG3873
Precorrin 4
cobM MSMEG3877
Precorrin 5
Rv2067c MSMEG5548
Precorrin
cobK 6x MSMEG3875
Precorrin 6y
cobL MSMEG3878
Precorrin 8x
cobH MSMEG3872
Hydrogenobyrinic acid
Co2+
cobB MSMEG2617
Cob(II)yrinic acid a,c-diamide
Hydrogenobyrinic acid a,c-diamide
cobN/Rv2850c MSMEG3864/2615
Rv0306/MSMEG6053
Adenosylcobyrinic acid a,c-diamide
cobO
Cob(I)yrinic acid a,c-diamide
cobQ1/cobQ2 MSMEG2588/6277
Adenosylcobyric acid
cobD MSMEG4310
corrin ring synthesis (CobI)
Riboflavin
ATP
ADP
FMN
(Flavin Mononucleotide)
FMN reductase
H+ NAD(P)H
NAD(P)+
FMNH2
(Reduced flavin mononucleotide)
Flavin destructase
SM: bluB
MTB:Rv0306
MSM:MEMEG6053
O2
H2O
DMB
+ D-erythrose-4-phosphate
L-threonine-3P
cobC
aminopropanol-2P
NaMN
DMB NaMN
cobT/
Rv1151c MSMEG4275
α-ribazole-5'-P
Rv2228c MSMEG4305
α-ribazole
α-ribazole synthesis (CobII)
Adenosylcobinamide
cobO
Cobinamide
cobU MSMEG4274
Adenosyl-cobinamide-GDP
cobS MSMEG4277
cobO/MSMEG2616
Cobalamin
Coenzyme B12
Adenosylcobalamin synthesis
(CobIII)
Corrinoid transport: unknown

Figure 3.9: Putative AdoCbl biosynthetic pathway in MTB and MSM incorporating the proposed pathway for DMB biosynthesis. The putative AdoCbl biosynthetic pathway from Figure 1.2 is expanded here to include the predicted pathway for DMB biosynthesis (shaded panel). H37Rv *Rv0306* (http://genolist.pasteur.fr/Tuberculist/) is shown together with predicted *S. meliloti* homologue *bluB*. The pathway is adapted from Metacyc reference pathway (http://metacyc.org/META/new-image?object=PWY-5523); a putative MSM homologue of *bluB* was identified by BLAST homology search (http://tigerblast.tigr.org/cmr.blast/) using *S. meliloti* query sequences.

Rv0306 was originally predicted to be a cob(II)yrinic acid a,c-diamide reductase ("cobalt reductase") in the B_{12} biosynthetic pathway (discussed in section 1.6.3). However, bioinformatic analyses (Rodionov *et al.*, 2003; Campbell *et al.*, 2006; Taga *et al.*, 2007), identified Rv0306 as the putative MTB homologue of *S. meliloti* BluB (Figure 3.9). Further examination of the Rv0306 protein sequence confirmed the conservation of two key residues required for BluB activity: the aspartate at position 32 and the glycine at position 167 (Figure 3.10). Taga *et al.* (2007) showed that mutations in these residues, either individually or in combination, abrogated DMB synthesis in *S. meliloti*. Although Rv0306 is annotated as an oxidoreductase, it is noteworthy that these residues are more commonly observed in nitroreductases, and the BluB enzyme belongs to a subfamily of the nitroreducatase family.

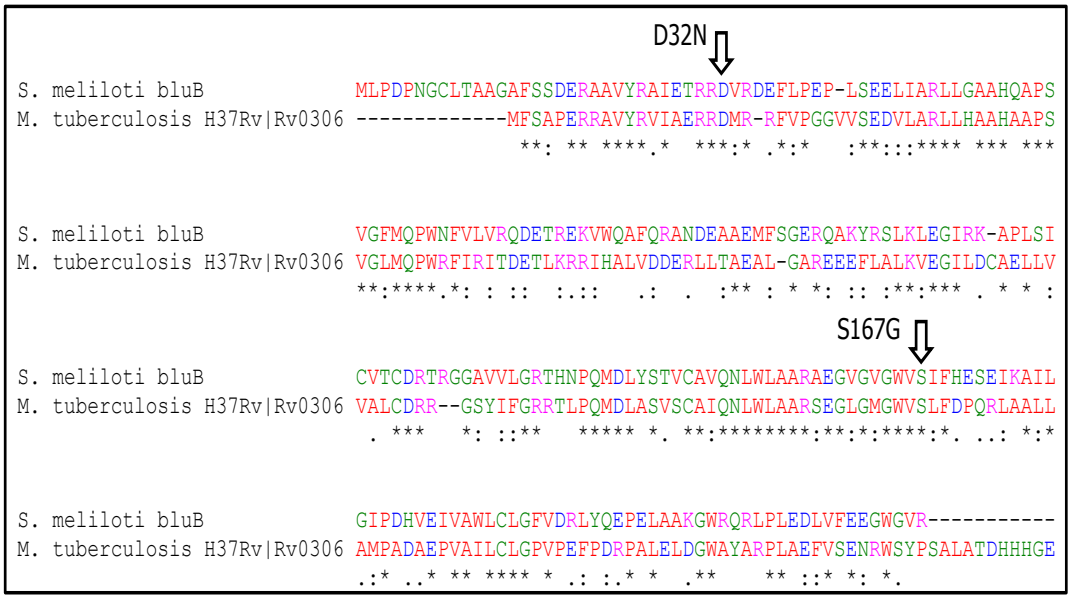

Figure 3.10: Sequence alignment of *S. meliloti* BluB and MTB Rv0306. Protein sequences were aligned utilizing the PROMALS3D multiple sequence and structure alignment server (http://prodata.swmed.edu/promals3d/promals3d.php). Critical residues mutated by Taga *et al.* (2007) are indicated by black arrows.

- ***Targeting of DMB biosynthesis in MTB by site-directed mutagenesis of key residues***

DMB biosynthesis in MTB was targeted by a twofold approach: (1) site-directed mutagenesis of the key residues in Rv0306 (Taga *et al.*, 2007), and (2) targeted knockout of *Rv0306*. The *Rv0306* allele was custom synthesized by Sigma and included the full-length Rv0306 plus substitutions of the key residues: that is, switching the aspartate at position 32 to asparagine (D32N) and glycine at position 167 to serine (S167G) (Figure 3.10). The mutant allele was synthesized with sufficient flanking sequence for homologous recombination and was utilized to construct the p2Rv0306^{D32NS167G}pG17 suicide vector (Table 2.2), which was electroporated into the MTB Δ*metE*::*hyg* mutant. The screening process applied to identify putative Rv0306^{D32NS167G}Δ*metE*::*hyg* mutants entailed first amplifying the 672bp *Rv0306* gene together with 190bp

upstream and 50 bp downstream sequence by PCR, and then digesting the resulting PCR product with *Fsp*I and *Stu*I restriction enzymes in order to exploit the restriction sites engineered into each point mutant (Figure 3.11A & B). Positive transformants harbouring both point mutations were confirmed by the presence of 203 bp and 651 bp fragments following *Fsp*I digest; and 597 bp plus 257 bp from the *Stu*I digest (Figure 3.11D & E). The deletion of the *metE* gene in the putative Rv0306^{D32NS167G} Δ*metE*::*hyg* mutant was re-confirmed by Southern blot (Figure 3.17C).

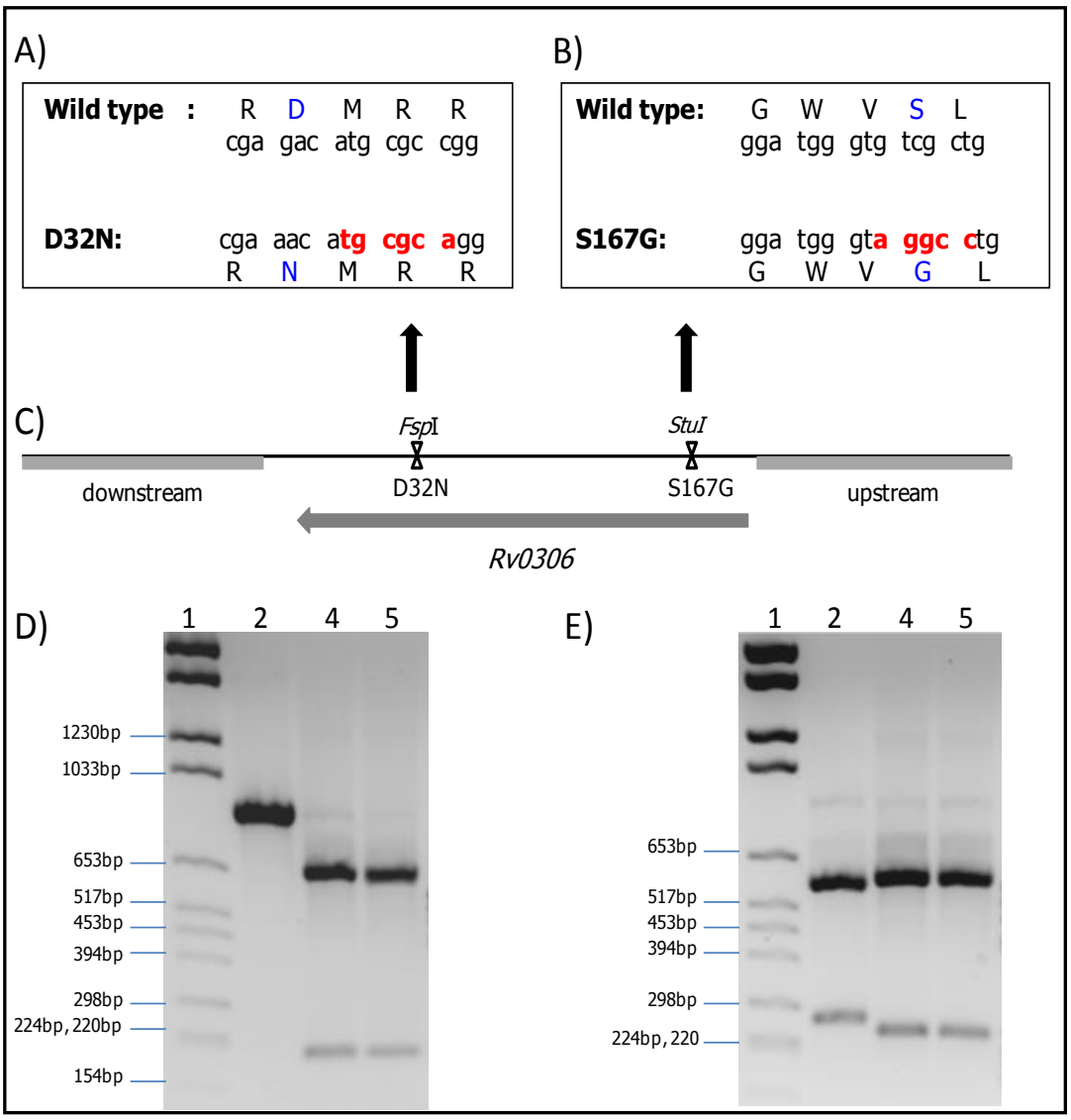

Figure 3.11: *Rv0306* with introduced point mutations. Protein sequences with corresponding nucleotide sequences showing that (A) the D32N mutation introduces *Fsp*I restriction site, and (B) the S167G mutation introduces *Stu*I restriction site, along with a (C) diagrammatic representation of Rv0306 illustrating point mutations and corresponding restriction sites. Restriction digests confirming point mutations in *Rv0306*, Lane 1: molecular weight marker VI (Roche), Lane 2: H37Rv, Lane 3: p2Rv0306^{D32NS167G}pG17, Lane 4: Rv0306^{D32NS167G}Δ*metE*::*hyg* (D) *Fsp*I digest confirming *Rv0306* with aspartate to asparagine point mutation. (E) *Stu*I digest confirming *Rv0306* with serine to glycine point mutation.

It was hypothesized that abrogation of DMB biosynthesis in Rv0306^{D32NS167G}Δ*metE*::*hyg* would render the mutant unable to grow on cobinamide alone and instead dependent on a combination of cobinamide plus DMB (to yield B_{12}) or cobinamide plus adenine (pseudo-B_{12}). However, as shown in Figure 3.12, the Rv0306^{D32NS167G} Δ*metE*::*hyg* mutant was able to grow when supplemented with cobinamide alone. The mutated residues had been selected based on the observations of Taga *et al.* (2007); the formal possibility remained, therefore, that the point mutations introduced into MTB *Rv0306* were not sufficient to abrogate BluB function and thus DMB biosynthesis.

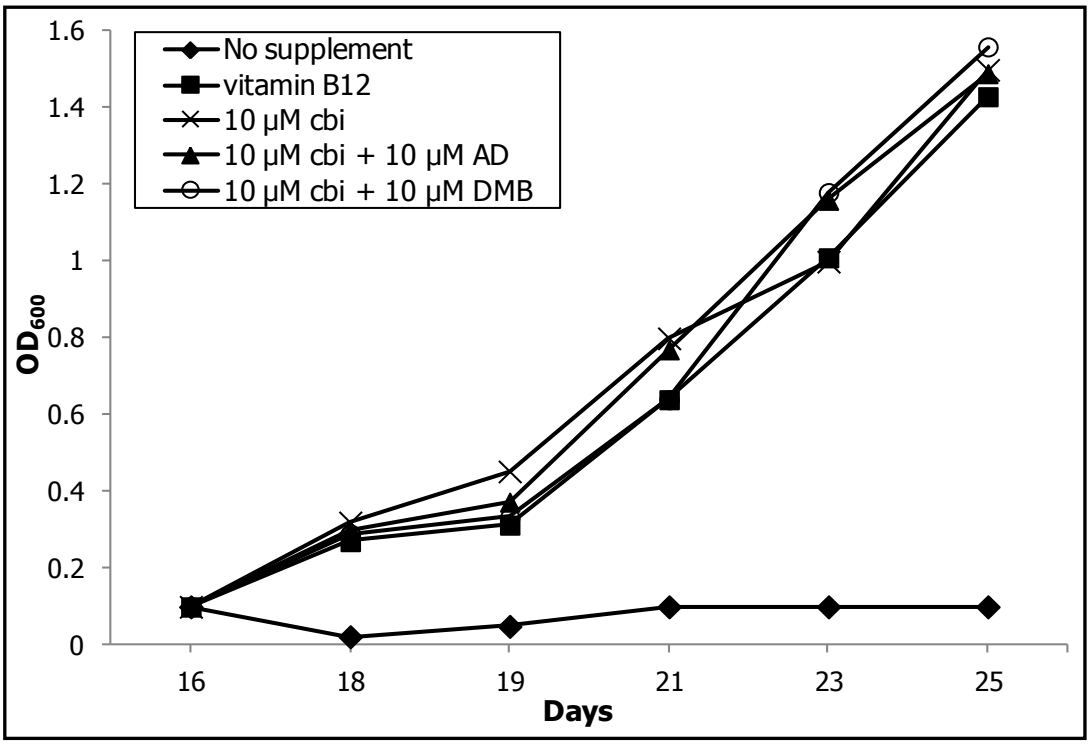

Figure 3.12: Mutation of Asp32 and Ser167 does not affect growth of MTB *Rv0306*^{D32NS167G} Δ*metE*::*hyg* on cobinamide. A log-phase culture of MTB *Rv0306*^{D32NS167G} Δ*metE*::*hyg* was washed then inoculated at an OD_{600} of 0.1 into Sauton's minimal medium, grown for 8-14 days until internal B_{12} was depleted, and then re-inoculated into medium containing exactly the same supplement(s). Growth of *Rv0306*^{D32NS167G} Δ*metE*::*hyg* in Sauton's medium with no supplement (◆), with B_{12} (10 µg/ml) (■), with 10 µM cobinamide (X), with 10 µM cobinamide plus 10 µM adenine (▲), and with 10 µM cobinamide plus 10 µM DMB (○). Data are representative of a single experiment from three independent biological replicates. Abbreviations: cbi - cobinamide, AD - adenine.

- ***Targeting of DMB biosynthesis by deletion of Rv0306***

Site-directed mutagenesis of two key residues in Rv0306 did not affect growth on cobinamide (Figure 3.12). To investigate the possibility that these mutations were insufficient to eliminate Rv0306 function, a double mutant was constructed by deleting the *Rv0306* gene in the Δ*metE*::*hyg* background (Figure 3.13). The deletion of *metE* in the Δ*Rv0306*

Δ*metE*::*hyg* double mutant was also confirmed genotypically (Figure 3.17C).

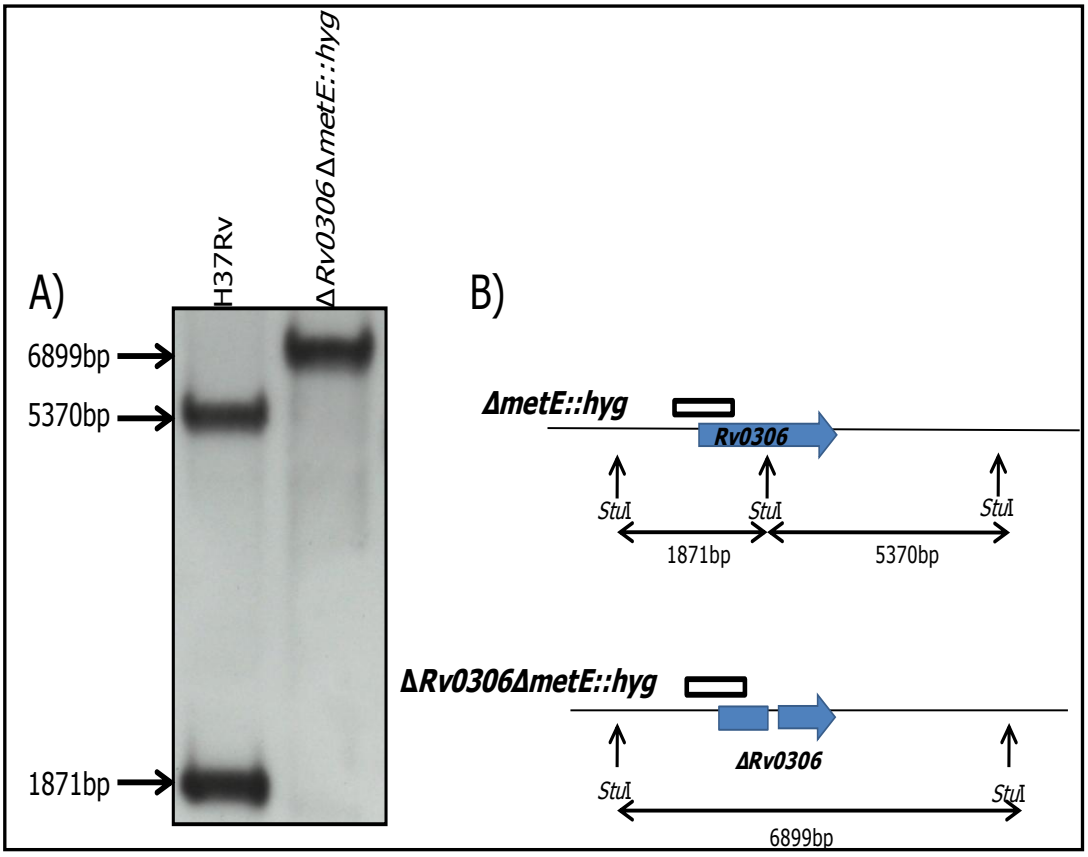

Figure 3.13: Genotypic analysis of the Δ*Rv0306* Δ*metE*::*hyg* double mutant. (A) Deletion of *Rv0306* in the Δ*metE* mutant background was confirmed by digesting genomic DNA isolated from H37Rv and Δ*Rv0306* Δ*metE*::*hyg* with restriction enzyme *Stu*I and probing with the *Rv0306* probe described in Table 2.3. Construction of the mutant allele eliminated 313bp of *Rv0306* coding sequence, and hybridization with the *Rv0306* probe identified 5370 bp and 1871 bp fragments in the parental H37Rv and 6899 bp fragment in the Δ*Rv0306* mutant allele. (B) Schematic representation of the parental and mutant allele plus restriction sites; the probe is represented by an open box.

The Δ*Rv0306* Δ*metE*::*hyg* double mutant grew with wild-type kinetics in Sauton's minimal medium supplemented with cobinamide alone (Figure 3.14). This result was surprising, and suggested a number of possibilities: for example, MTB might possess an alternative pathway(s) for DMB biosynthesis, or *Rv0306* might not be the only enzyme capable of catalysing the flavin destructase reaction. Alternatively, it is possible that an alternate form of B_{12} is synthesized by MTB using an endogenous α-ligand, such as adenine. This result reinforced the conclusion that detection of B_{12} or pseudo-B_{12} synthesis was not possible utilizing a genetic assay alone, and suggested that a more precise biochemical assay would be required to determine the nature of the active cofactor.

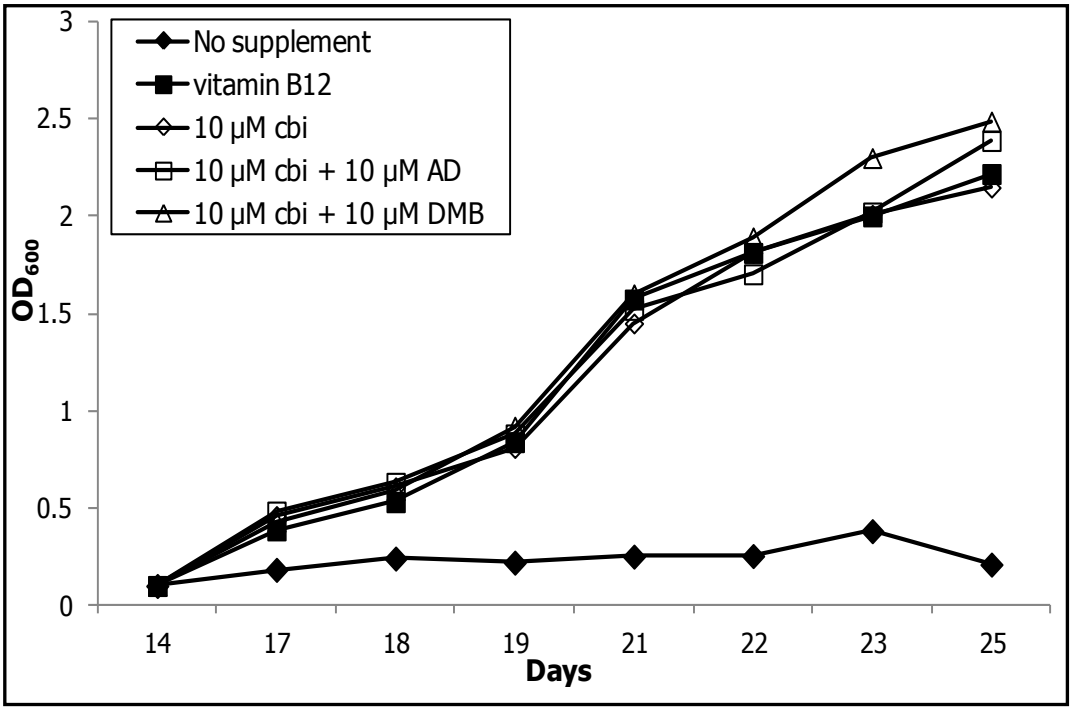

Figure 3.14: Phenotypic analysis of the Δ*Rv0306* Δ*metE*::*hyg* double mutant. Δ*Rv0306* Δ*metE*::*hyg* was phenotypically assessed by growing to log-phase then washing and inoculating the culture at an OD_{600} of 0.1 into Sauton's minimal media, with no supplement (◆), with B_{12} (10 μg/ml) (■), with 10 μM cobinamide (◇), with 10 μM cobinamide plus 10 μM adenine (□), and with 10μM cobinamide plus 10 μM DMB (△). Cultures were grown for 8-14 days until internal B_{12} was depleted, then re-inoculated into media containing the same supplement(s) and then OD's were taken. Data are representative of a single experiment from three independent biological replicates. Abbreviations: cbi- cobinamide, AD- adenine.

3.3 Cobalt supplementation enables growth of a MTB B_{12} auxotroph

MTB encodes a near-complete B_{12} biosynthetic pathway (discussed in section 1.6.3), and appears to lack homologues of *cobF* and *cobST*. However, B_{12} biosynthesis has been microbiologically demonstrated in the closely related *M. bovis* (Karasseva *et al.*, 1977) which, like MTB, appears to lack CobF and CobST but, unlike MTB, also possesses a truncated CobL (Brosch *et al.*, 2002). Therefore, the inability of the Δ*metE*::*hyg* mutant (Warner *et al.*, 2007) to grow without B_{12} supplementation (Figure 3.15A) was intriguing.

Industrial production of B_{12} by various bacterial species is influenced by a variety of conditions; for example, the addition of key precursors to the culture medium has been shown to increase B_{12} yields (Halbrook *et al.*, 1950; Kamikubo *et al.*, 1978; Riaz *et al.*, 2007). Some of these precursors (as seen in section 3.2) also facilitated synthesis of B_{12} in MTB, but the results did not address the question of whether the bacillus is capable of corrin ring synthesis. At the heart of the corrin macrocycle lies a cobalt ion; the supplementation of growth media with cobalt is an absolute requirement in the industrial synthesis of B_{12}, as it enhances yields.

Therefore, it was speculated that cobalt availability might be a limiting factor for MTB B_{12} biosynthesis under standard *in vitro* conditions. To investigate this possibility, the Δ*metE*::*hyg* mutant was grown in Sauton's minimal medium containing cobalt. As shown in Figure 3.15B, 5 μM cobalt can supplement growth of the MTB B_{12} auxotroph. Although B_{12} biosynthesis had not been demonstrated biochemically, this was a significant result since it suggested corrin ring synthesis in MTB.

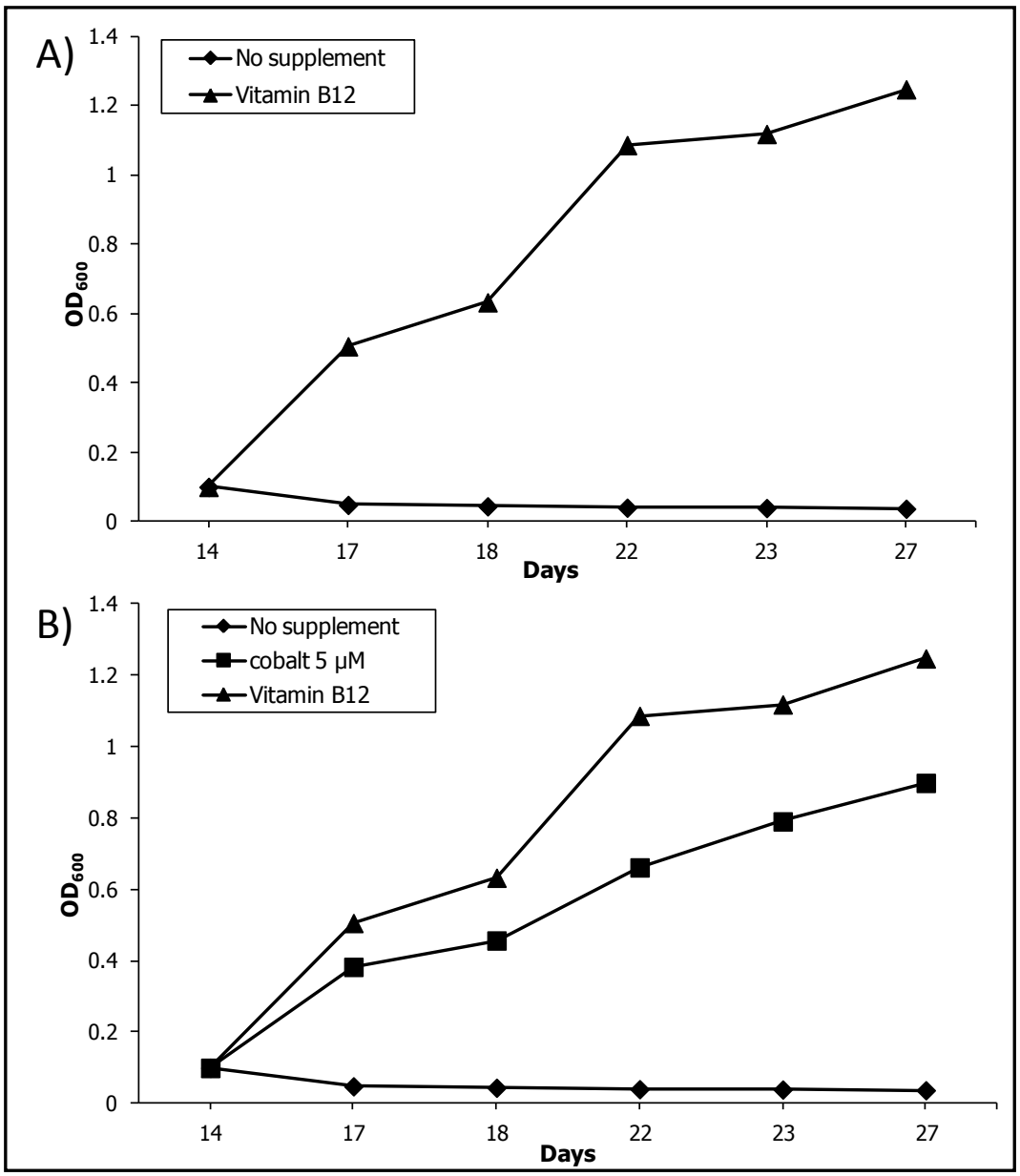

Figure 3.15: Exogenous cobalt supports growth of MTB B_{12} auxotroph. A log-phase culture of the Δ*metE*::*hyg* mutant was washed then inoculated at an OD_{600} of 0.1 in Sauton's minimal medium, and grown for 8 to 14 days to allow depletion of internal B_{12}. Cultures were re-inoculated into fresh medium containing the identical supplement, and optical densities measured. Growth of Δ*metE*::*hyg* mutant in minimal medium supplemented with (A) 10 µg/ml B_{12}, and (B) 5 µM cobalt. Data are representative of a single experiment from three independent biological replicates.

The near-complete B_{12} biosynthetic pathway in MTB exhibits characteristics of aerobic biosynthesis. As a result, the insertion of cobalt into the corrin macrocycle is predicted to occur late in the pathway. To confirm the notion that cobalt was limiting in standard growth media, and that supplementation with cobalt enabled *de novo* B_{12} biosynthesis in MTB, a panel of mutants was constructed containing disruptions in key B_{12} biosynthetic genes. An early step in the pathway that involves the conversion of precorrin-6x to precorrin-6y is catalyzed by the CobK-encoded precorrin-6x reductase (Figure 3.9). Deletion of *cobK* in the Δ*metE*::*hyg* background eliminated the ability of the resulting Δ*cobK* Δ*metE*::*hyg* double mutant (Figure 3.17A & C) to grow in minimal medium supplemented with cobalt (Figure 3.16A). In contrast, supplementation with cobinamide, a late-stage product of the pathway, enabled growth (Figure 3.16A)

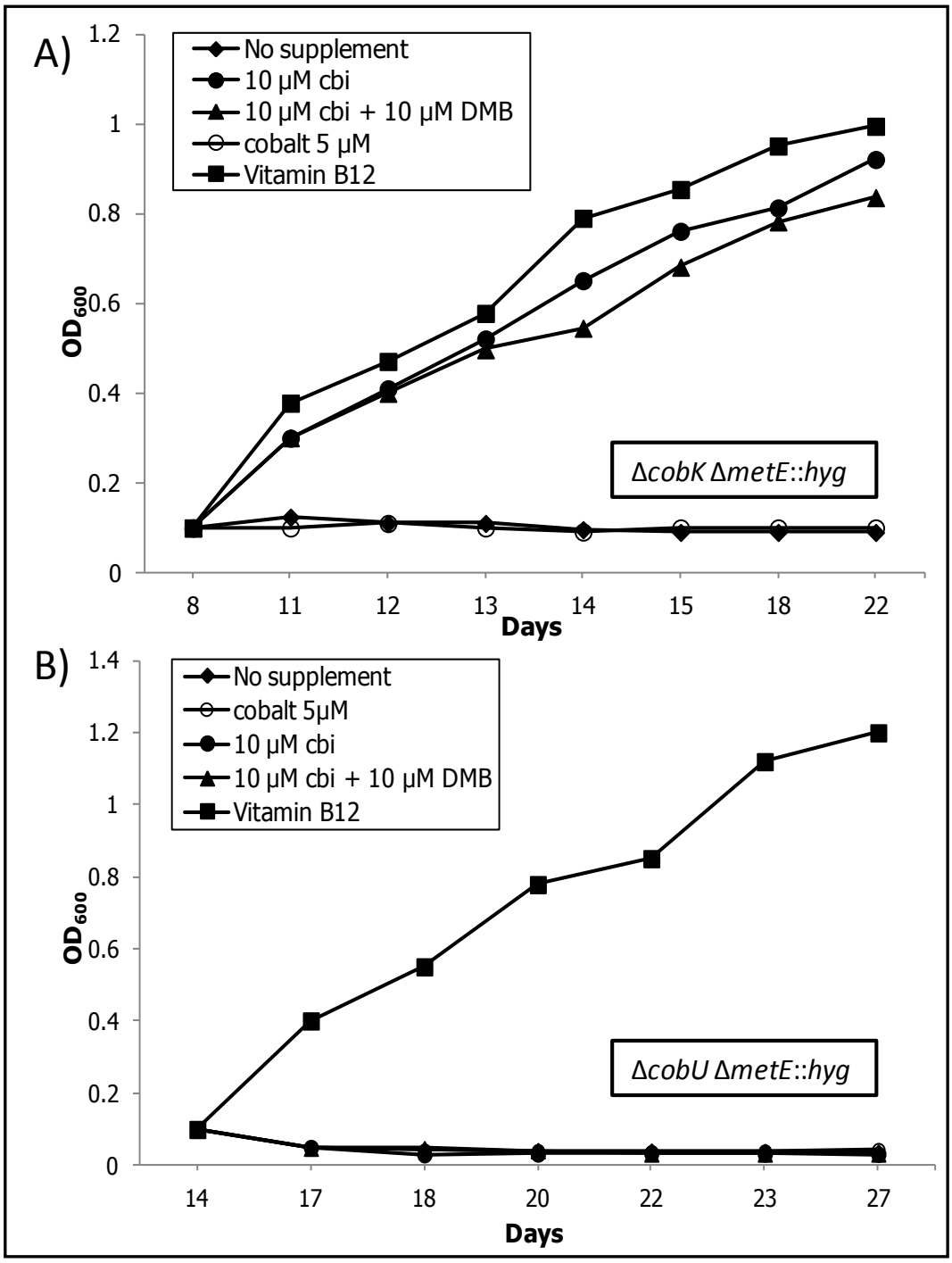
A)
No supplement
10 µM cbi
10 µM cbi + 10 µM DMB
cobalt 5 µM
Vitamin B12
ΔcobK ΔmetE::hyg
OD600
Days
B)
No supplement
cobalt 5µM
10 µM cbi
10 µM cbi + 10 µM DMB
Vitamin B12
ΔcobU ΔmetE::hyg
OD600
Days

Figure 3.16: Abrogation of predicted early- (CobK) and late-stage (CobU) vitamin B_{12} biosynthetic steps in MTB Δ*metE*::*hyg* suggests the incorporation of cobalt into vitamin B_{12}. Log-phase cultures of Δ*cobK* Δ*metE*::*hyg* and Δ*cobU* Δ*metE*::*hyg* were washed then inoculated at an OD_{600} of 0.1 into Sauton's minimal medium, and grown for 8 to 14 days to allow internal B_{12} depletion. Cultures were re-inoculated into fresh media containing the same supplement and optical densities were measured. (A) Inability of Δ*cobK* Δ*metE*::*hyg* to grow in unsupplemented medium (◆) and medium containing 5 μM cobalt (○) contrasts with growth of this mutant in medium supplemented with 10 μM cobinamide (●), 10 μM cobinamide plus 10 μM DMB (▲), and 10 μg/ml B_{12} (■). (B) The Δ*cobU* Δ*metE*::*hyg* mutant is able to grow only when supplemented with 10 μg/ml B_{12}, but not in medium supplemented with 5 μM cobalt (○), 10 μM cobinamide (●), or 10 μM cobinamide plus 10 μM DMB (▲). Data are representative of a single experiment from three independent biological replicates. Abbreviations: cbi- cobinamide, AD- adenine.

One of the final steps in B_{12} biosynthesis involves the conversion of adenosylcobinamide to adenosyl-cobinamide-GDP in a reaction catalyzed by the bifunctional enzyme, CobU (Figure 3.9). This enzyme represents a crucial step in the synthesis of B_{12} because it functions as both a kinase and guanylyltransferase in AdoCbl biosynthesis. To investigate the effects of late-stage disruptions on the ability of the Δ*metE*::*hyg* mutant to utilize exogenous cobalt, a double mutant was constructed (Warner, 2006) by deleting *metE* in the *cobU* background (Figure 3.17B & C). In contrast to Δ*cobK* Δ*metE*::*hyg* (Figure 3.16A), the resulting Δ*cobU* Δ*metE*::*hyg* double mutant was unable to grow in minimal medium supplemented with either cobalt or cobinamide (alone or plus DMB) (Figure 3.16B). This strongly supports the conclusion that cobalt is incorporated into vitamin B_{12}, and reinforces the idea that supplementation with cobalt is necessary to enable *de novo* B_{12} biosynthesis by MTB *in vitro.* In addition, it suggests that the full B_{12} biosynthetic pathway is functional in MTB, despite the absence of clear homologoues for every biosynthetic step (discussed in 1.6.3).

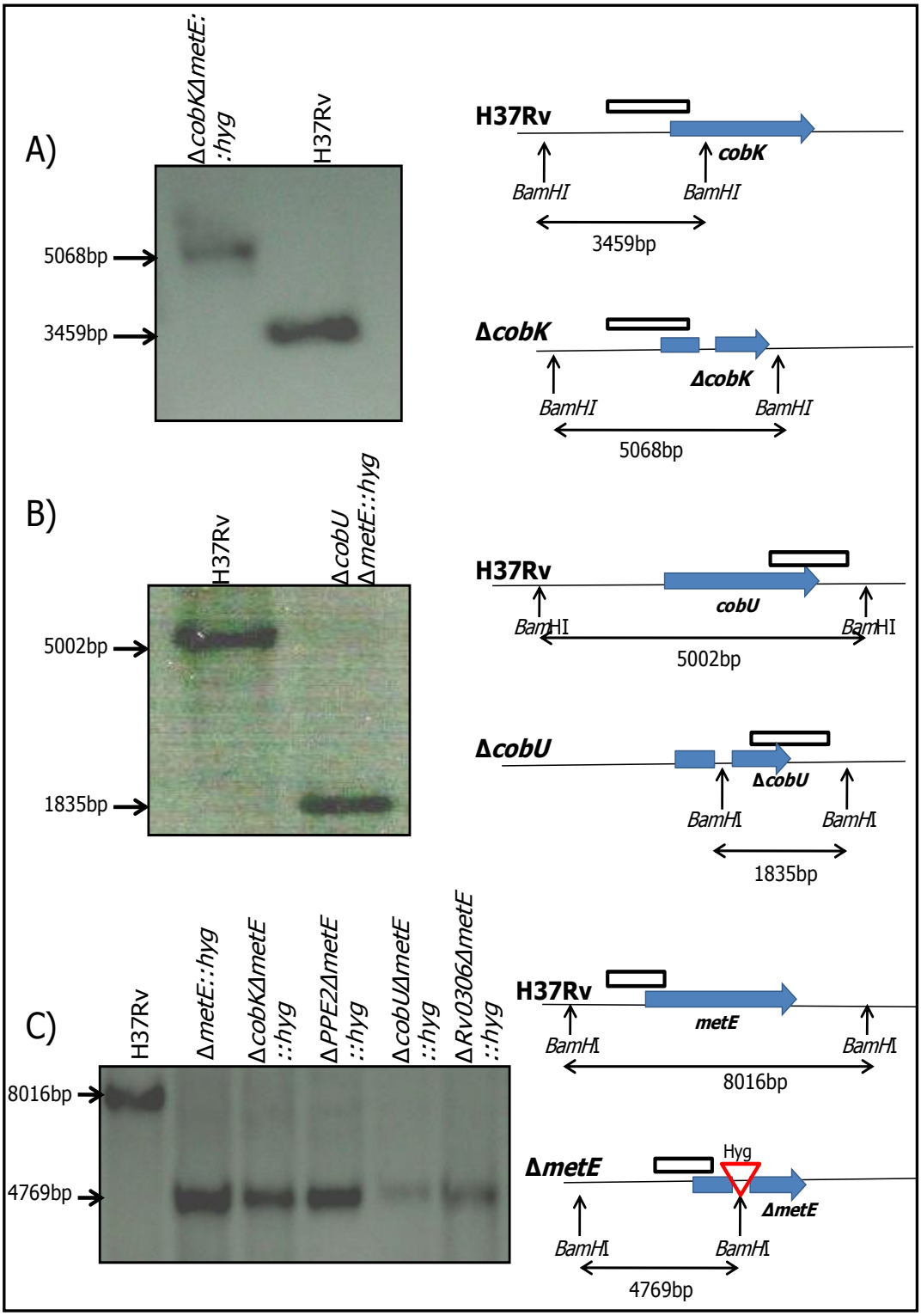
A)
ΔcobKΔmetE::hyg
H37Rv
5068bp
3459bp
H37Rv
cobK
BamHI
BamHI
3459bp
ΔcobK
ΔcobK
BamHI
BamHI
5068bp
B)
H37Rv
ΔcobU ΔmetE::hyg
5002bp
1835bp
H37Rv
cobU
BamHI
BamHI
5002bp
ΔcobU
ΔcobU
BamHI
BamHI
1835bp
C)
H37Rv
ΔmetE::hyg
ΔcobKΔmetE ::hyg
ΔPPE2ΔmetE ::hyg
ΔcobUΔmetE ::hyg
ΔRv0306ΔmetE ::hyg
8016bp
4769bp
H37Rv
metE
BamHI
BamHI
8016bp
ΔmetE
Hyg
ΔmetE
BamHI
BamHI
4769bp

Figure 3.17: Genotypic confirmation of methionine and B_{12} deletion mutants. Genomic DNA was isolated from H37Rv, Δ*metE*::*hyg*, Δ*cobK* Δ*metE*::*hyg*, Δ*PPE2* Δ*metE*::*hyg*, Δ*cobU* Δ*metE*::*hyg*, and Δ*Rv0306* Δ*metE*::*hyg* and digested with restriction enzyme *Bam*HI before probing with specific *metE*, *cobK* and *cobU* probes, listed in Table 2.3. Adjacent to each Southern blot is a diagrammatic representation of parental and mutant allele plus restriction sites. The probe is represented by an open black box in each case. (A) Construction of the Δ*cobK* allele eliminated 214 bp of *cobK* coding sequence and hybridization with *cobK* probe identified a 5068 bp fragment in the parental H37Rv and a 3459 bp fragment in the Δ*cobK* mutant. (B) Construction of the Δ*cobU* allele eliminated 280 bp of *cobU* coding sequence and introduced an additional *Bam*HI restriction site in the deletion allele. Hybridization with the *cobU* probe identified a 5002 bp band in the parental H37Rv and a 1835 bp band in the Δ*cobU* mutant. (C) Construction of the Δ*metE*::*hyg* allele (*hyg* cassette represented by red triangle) eliminated 1367 bp of *metE* coding sequence and introduced an additional *Bam*HI restriction site in the deletion allele. Hybridization with the *metE* probe identified an 8016 bp fragment in the parental strain and a 34769 bp band in the Δ*metE*::*hyg* mutant.

3.3.1 Genetic complementation of late stage (cobU) B_{12} auxotroph with PPE2-cobQ1-cobU

The *cobU* gene lies in a putative three-gene operon with *PPE2* and *cobQ1* (Tundup *et al.,* 2006*)* and is only 525 bp in size. A construct carrying a wild-type copy of the putative operon (*PPE2-cobQ1-cobU*) plus 688 bp 5' upstream sequence was integrated at the *attB* site of the Δ*cobU* Δ*metE*::*hyg* double mutant. As shown in Figure 3.18, complementation with this construct restored the ability of the double mutant to grow in medium supplemented with either cobalt or cobinamide, strongly suggesting that abrogation of CobU function eliminates the ability of MTB to synthesize a viable cofactor.

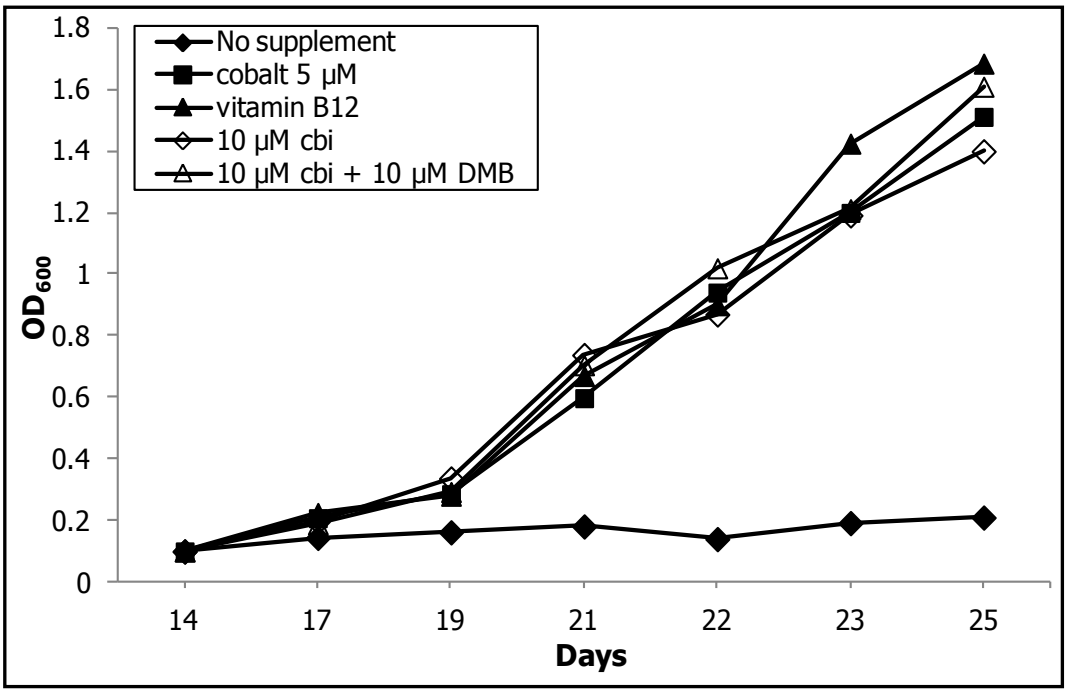

Figure 3.18: Complementation with *PPE2-cobQ1-cobU* restores growth of Δ*cobU* Δ*metE*::*hyg* on B_{12} precursors. A log-phase culture of Δ*metE*::*hyg* Δ*cobU attB*::*PPE2-cobQ1-cobU* was washed then inoculated at an OD_{600} of 0.1 into Sauton's minimal medium, and grown for 8 to 14 days to allow internal B_{12} depletion. The culture was re-inoculated into fresh media containing the same supplement and optical densities were measured. Δ*metE*::*hyg ΔcobU attB*::*PPE2-cobQ1-cobU* is unable to grow in unsupplemented (◆) medium, but growth is restored in medium containing 5 µM cobalt (■), 10 µM cobinamide (◇), 10 µM cobinamide plus 10 µM DMB (△), and 10 µg/ml B_{12} (▲). Data are representative of a single experiment from three independent biological replicates. Abbreviations: cbi-cobinamide, AD-adenine.

3.3.2 Inability to supplement the ΔmetE::hyg mutant with cobalt on solid medium

To determine whether cobalt supplementation was similarly effective on solid medium, the Δ*metE*::*hyg* mutant was plated on 7H10/OADC plates containing increasing concentrations of cobalt (Figure 3.19). Surprisingly, even the highest concentration of cobalt failed to complement growth of the Δ*metE*::*hyg* mutant (Figure 3.15B). In contrast, cobinamide was able to support growth of the Δ*metE::hyg* mutant on solid medium (data not

shown). The reason for the failure of the Δ*metE*::*hyg* mutant to utilize exogenous cobalt for growth on solid media is unclear; however, it is possible that uptake of the metal ion – or even the synthesis of B_{12} - is inefficient and/or impaired under these conditions.

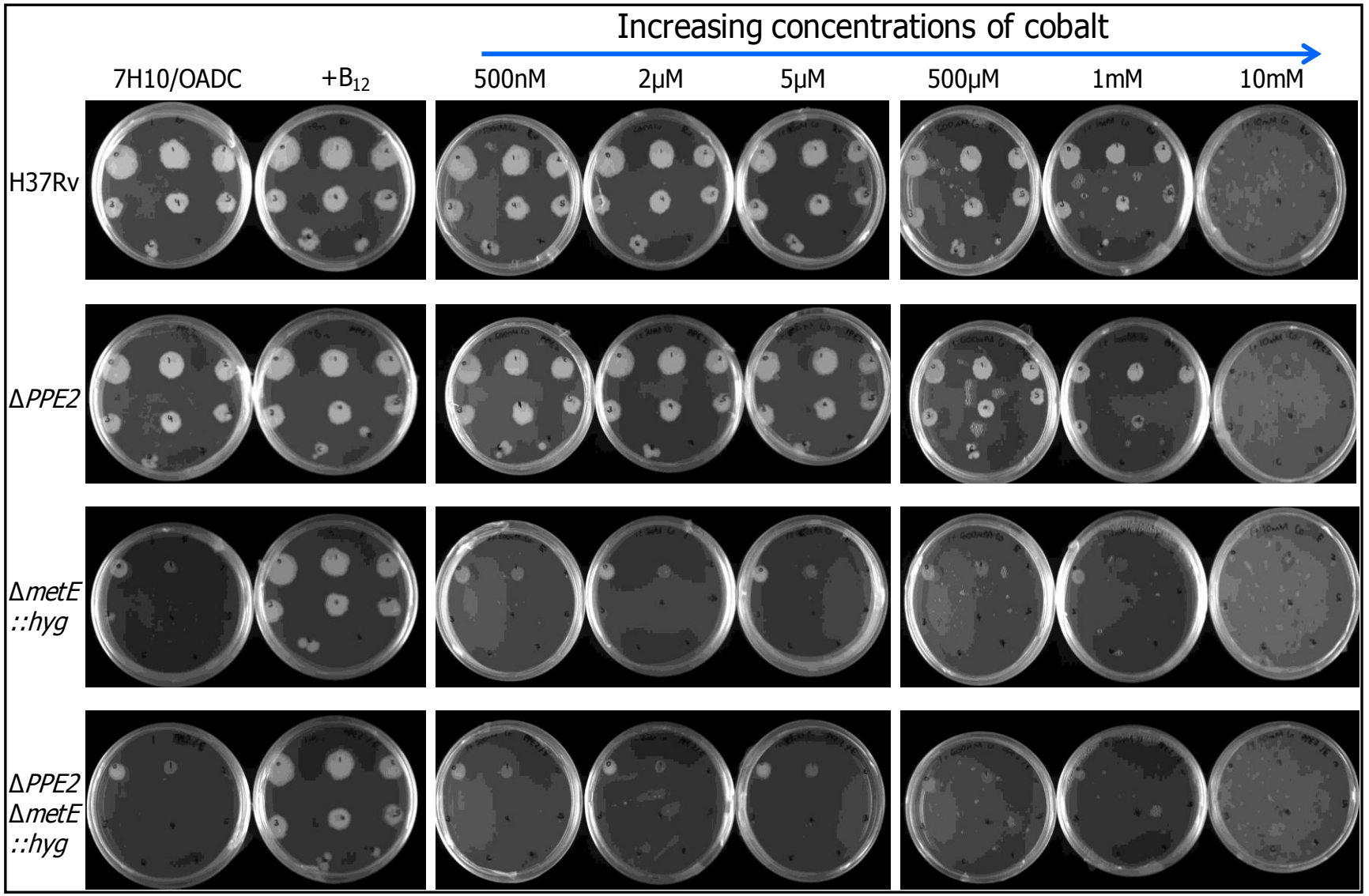
Increasing concentrations of cobalt
7H10/OADC
+B12
500nM
2µM
5µM
500µM
1mM
10mM
H37Rv
ΔPPE2
ΔmetE ::hyg
ΔPPE2 ΔmetE ::hyg

Figure 3.19: Inability of cobalt to complement growth of the Δ*metE*::*hyg* mutant on solid medium. The effect of increasing concentrations of cobalt on growth of H37Rv, Δ*PPE2*, Δ*metE*::*hyg* and Δ*PPE2* Δ*metE*::*hyg* was assessed by spotting 10 µl of a 10-fold serial dilution of log phase cultures of each strain on 7H10/OADC supplemented with 500 nM, 2 µM, 5 µM, 500 µM, 1 mM and 10 mM cobalt. Unsupplemented 7H10/OADC and vitamin B_{12}-supplemented 7H10/OADC plates were included as controls.

3.4 Investigation of the putative role of PPE2 in B_{12} or cobalt transport/assimilation

In addition to providing evidence of MTB's inability to synthesize B_{12} (in the absence of cobalt supplementation), the B_{12} auxotrophy of the *metE* mutant (Warner *et al.*, 2007) established that MTB is able to transport B_{12} (Figure 3.15A). This was an important finding; unlike the majority of bacteria that transport B_{12}, there is a complete lack of any identifiable B_{12}-specific transporter in the MTB genome (Rodionov *et al.*, 2003). Of the genomes analysed by Rodionov *et al.* (2003), only a small proportion of B_{12}-utilizing organisms lack an obvious B_{12} transport candidate, including MTB. However, the bioinformatic analysis conducted by Rodionov and colleagues (2003) did identify a putative *cbtG*-type B_{12}-regulated cobalt transporter in MTB. According to their results, this function was encoded by *PPE2*.

The PE/PPE's belong to a large family of mycobacterium-specific proteins that consume almost 10% of the coding potential of the MTB genome but whose functional characterization remains elusive (Cole *et al.*, 1998). Of the more than sixty *PPE* genes in MTB, *PPE2* belongs to the PPW sub-family (Van Pittius *et al.*, 2006). As noted previously, PPE2 is the first gene in a predicted operon comprising the B_{12} biosynthetic genes, *cobQ1* and *cobU*. It is also unusual in that its 5' region contains one of only two B_{12}-

riboswitches in the MTB genome. The riboswitch is located 133 bp upstream of the predicted PPE2 start codon. As noted in the Introduction (section 1.10), B_{12}-riboswitches typically occur upstream of genes involved in transport of B_{12} or cobalt (Richter-Dahlfors and Andersson, 1992; Cheng *et al.*, 2011), genes required for B_{12} biosynthesis (Lundrigan *et al.*, 1991), or B_{12}-independent enzymes with corresponding B_{12}-dependent alternatives (Borovok *et al.*, 2006; Warner *et al.*, 2007). Therefore, the presence of a B_{12}-riboswitch upstream of the *PPE2-cobQ1-cobU* locus was strongly suggestive of the involvement of PPE2 in a B_{12}-related pathway (Figure 3.9). In light of these observations, the third major component of this study comprised an investigation of the potential ability of PPE2 to function in B_{12} or cobalt transport/assimilation in MTB.

3.4.1 Assessment of a role for PPE2 in B_{12} transport in MTB

- ***Transcriptional analysis of* metE *in the* Δ*PPE2 mutant***

To investigate the role of *PPE2* in B_{12} transport, a targeted knockout was generated (Figure 3.22A). No difference in growth was observed between H37Rv and Δ*PPE2* both in the presence and absence of B_{12} (data not shown). This was expected since the only B_{12}-dependent phenotype identified to date in standard laboratory medium results from impaired methionine biosynthesis (Figure 3.15A); in the Δ*PPE* mutant, the B_{12}-independent methionine synthase, *metE*, is still intact. Therefore, it was hypothesized that if *PPE2* were involved in B_{12} transport, the B_{12}-mediated down regulation of *metE* (Warner *et al.*, 2007) would not occur in the Δ*PPE2* mutant. Expression of *metE* was determined in vitamin B_{12}-supplemented 7H9/OADC medium using untreated control samples for each time point and normalizing results against *sigA* expression. Semi-quantitative transcriptional data in Figure 3.20 indicate that *metE*

transcript levels were increased in the absence of B_{12} and notably diminished in the presence of B_{12} after 10 h, as was previously demonstrated in wild-type MTB (Warner *et al.*, 2007). This observation suggested that vitamin B_{12} was being accessed by the Δ*PPE2* mutant and, in turn, this suggested that PPE2 might not have a role in the transport of this cofactor.

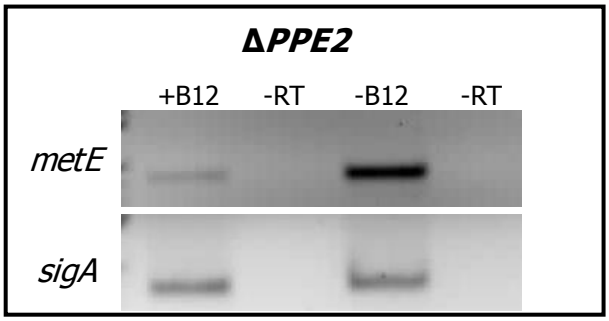

Figure 3.20: RT-PCR analysis of *metE* expression in a Δ*PPE2* mutant in response to B_{12}. Cells were grown for 10 h in 7H9/OADC in the presence (+B_{12}) or absence (-B_{12}) of 10 µg/ml vitamin B_{12} before harvesting for mRNA extraction. The *sigA* housekeeping gene and no reverse transcriptase (-RT) controls were included.

- ***Assessment of the role of PPE2 in B_{12} transport utilizing growth on valerate as a carbon source***

Subsequent to the inception of this study, a simple assay (described in section 3.2.4) for growth of MTB on odd-chain fatty acids presented the option of phenotypically differentiating the Δ*PPE2* mutant from H37Rv. To investigate the possibility that *PPE2* functions in B_{12} transport, the growth of the Δ*PPE2* mutant was compared with wild-type H37Rv in the presence of 3-NP where valerate constituted the sole carbon source (Savvi *et al.*, 2008). The underlying rationale for this experiment was that, were *PPE2* the MTB B_{12} transporter, then a Δ*PPE2* mutant should not be able to grow on valerate in the presence of 3-NP, even if supplemented with B_{12}.

Growth of the Δ*PPE2* mutant in valerate as a sole carbon source was comparable to that of H37Rv, as illustrated in Figure 3.21A. Wild-type MTB was unable to utilize valerate as sole carbon source in media containing 3-NP (Figure 3.21B) in accordance with observations made by Savvi *et al.* (2008). However, supplementing media with B_{12} restored growth. Notably, the Δ*PPE2* mutant achieved wild-type growth levels under the same B_{12}-supplemented conditions, suggesting that *PPE2* does not function in B_{12} transport in MTB (Figure 3.21B).

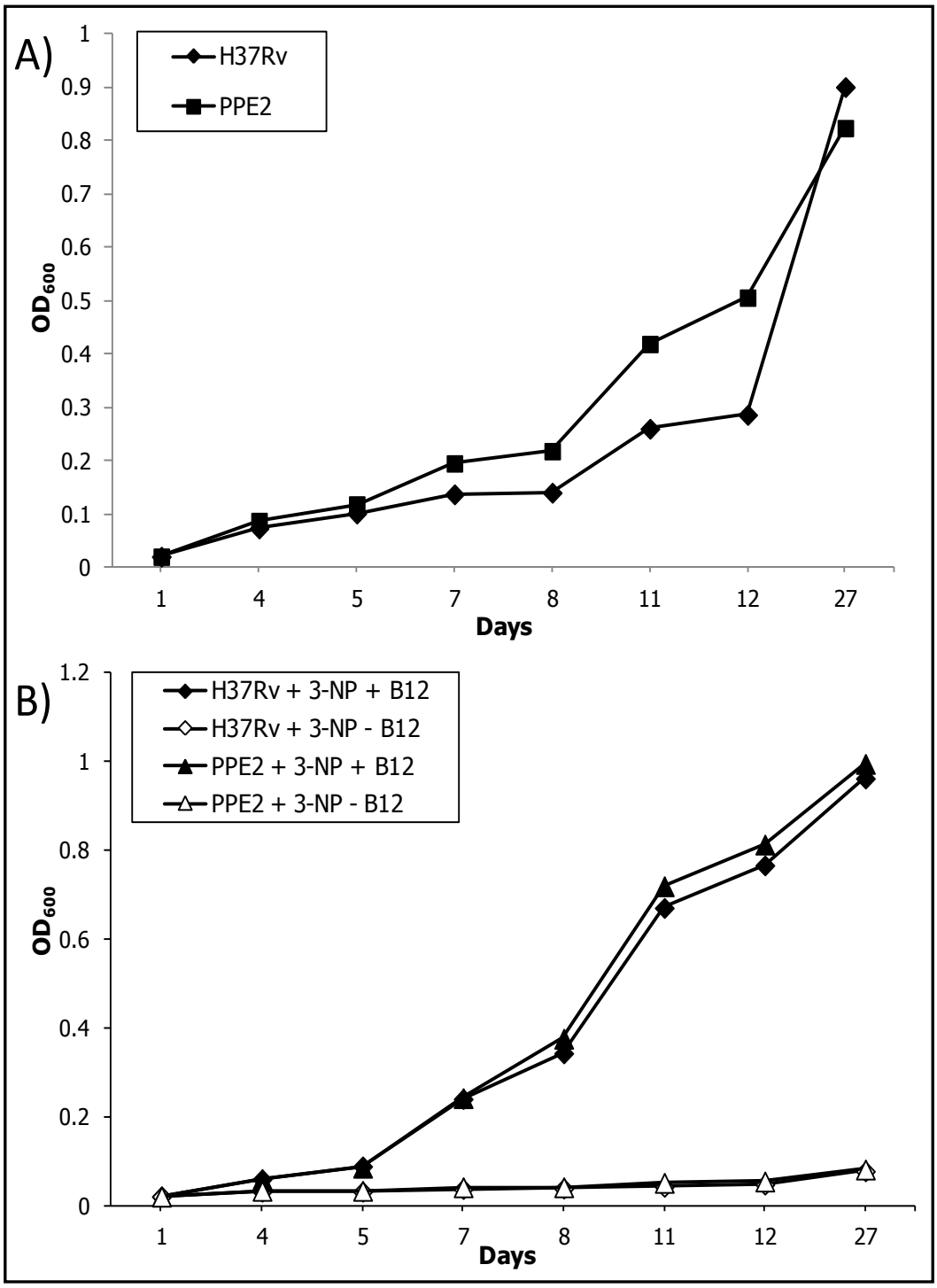
A)
H37Rv
PPE2
OD600
Days
B)
H37Rv + 3-NP + B12
H37Rv + 3-NP - B12
PPE2 + 3-NP + B12
PPE2 + 3-NP - B12
OD600
Days

Figure 3.21: Growth kinetics of the Δ*PPE2* mutant in valerate with 3-NP and B_{12}. (A) H37Rv and the Δ*PPE2* mutant were grown in medium containing valerate as the sole carbon source, (B) H37Rv with 3-NP and vitamin B_{12} (◆) or without vitamin B_{12} (◇), versus growth of the Δ*PPE*2 strain on valerate with 3-NP and vitamin B_{12} (▲), or without vitamin B_{12} (△). Data are representative of a single experiment from three independent biological replicates.

3.4.2 Assessment of the role of PPE2 in cobalt transport/assimilation

The results above demonstrated that PPE2 does not have a role in B_{12} transport in MTB. However, the possibility remained that PPE2 might function in cobalt transport, as predicted by Rodionov *et al.* (2003). Therefore, to investigate the potential role of PPE2 in cobalt transport/assimilation in MTB, an assay was developed that exploited the ability of cobalt to complement the Δ*metE*::*hyg* mutant (Figure 3.15B). To this end, a targeted knockout of *PPE2* was generated in the Δ*metE*::*hyg* background to yield a Δ*PPE2* Δ*metE*::*hyg* double mutant (Figure 3.17C & Figure 3.22A). This strain was unable to grow in unsupplemented liquid medium (Figure 3.22B), and required the addition of exogenous vitamin B_{12} for replication. In contrast to the parental *metE* strain (Figure 3.15B), the Δ*PPE2* Δ*metE*::*hyg* double mutant was unable to grow in medium containing cobalt alone (Figure 3.22B), strongly suggesting a role for PPE2 in cobalt/transport assimilation in MTB.

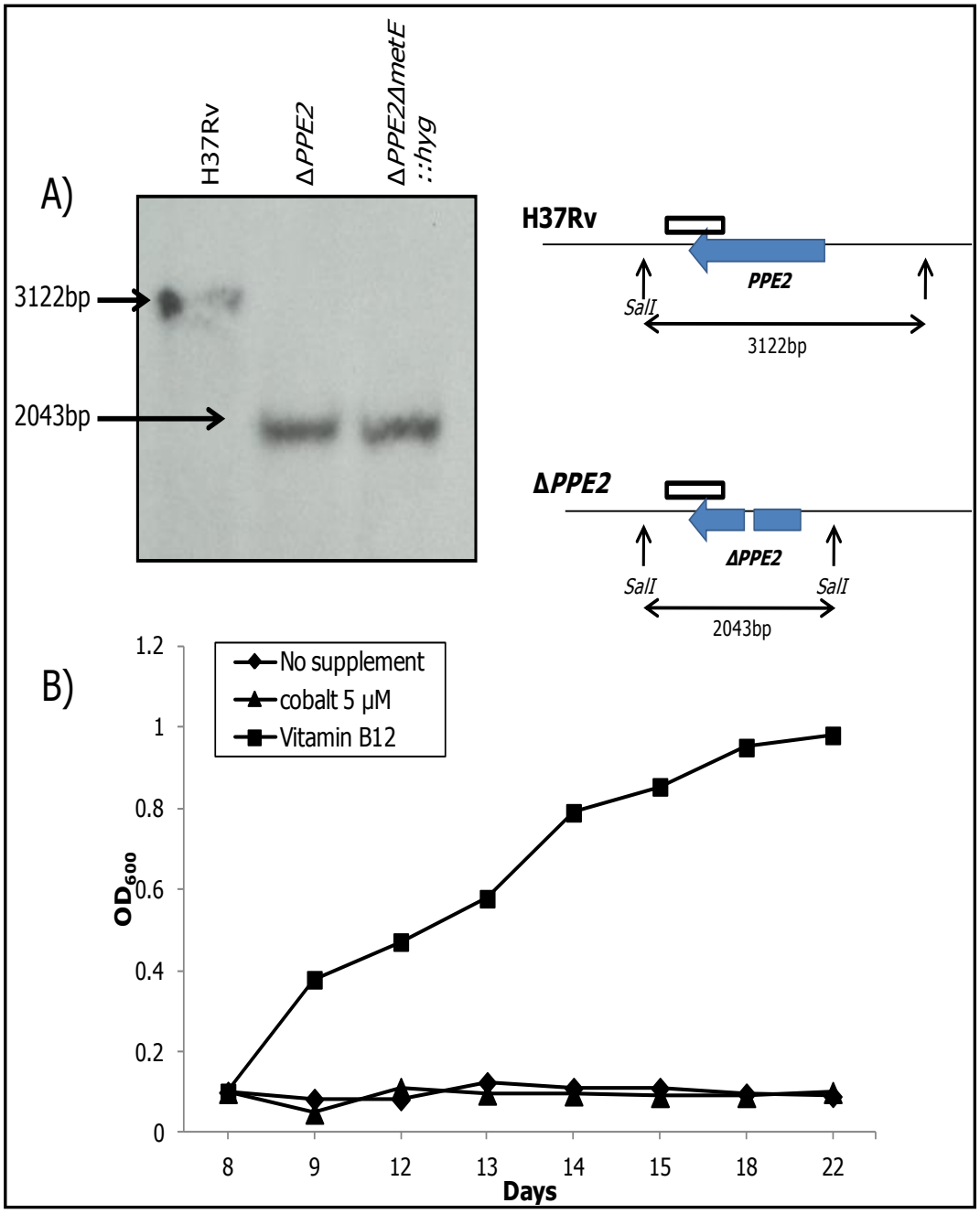
A)
H37Rv
ΔPPE2
ΔPPE2ΔmetE ::hyg
3122bp
2043bp
H37Rv
PPE2
SalI
3122bp
ΔPPE2
ΔPPE2
SalI
SalI
2043bp
B)
No supplement
cobalt 5 µM
Vitamin B12
OD600
1.2
1
0.8
0.6
0.4
0.2
0
8
9
12
13
14
15
18
22
Days

Figure 3.22: Genotypic and phenotypic characterization of *PPE2* deletion in H37Rv and the Δ*metE*::*hyg* mutant. (A) Southern blot analysis of genomic DNA isolated from H37Rv, Δ*PPE2* and Δ*PPE2* Δ*metE*::*hyg* digested with restriction enzyme, *SalI.* Hybridization with PPE2 probe (Table 2.3) identified a 3122 bp fragment in the parental H37Rv and 2043 bp in deleted *PPE2* gene. Adjacent to the Southern blot is the schematic representation of parental and mutant allele illustrating restriction enzyme sites with PPE2 probe represented by black box. The *PPE2* deletion allele eliminated 1100 bp of internal *PPE2* coding sequence. (B) Log-phase culture of Δ*PPE2* Δ*metE*::*hyg* mutant was washed then inoculated at an OD_{600} of 0.1 into Sauton's minimal media, and grown for 8 to 14 days to allow depletion of internal B_{12}. Culture was re-inoculated into fresh media containing the same supplement(s) and optical densities were measured. The Δ*PPE2* Δ*metE*::*hyg* double mutant was unable to grow when supplemented with cobalt (5 μM). Data are representative of a single experiment from three independent biological replicates.

As *PPE2* appears to lie in a putative operon with key B_{12} biosynthetic genes, *cobQ1* and *cobU*, complementation of the *PPE2* deletion was initially performed by integrating a wild-type copy of the putative operon (*PPE2-cobQ1-cobU*) plus 688 bp 5' sequence at the *attB* locus. As shown in Figure 3.23A, complementation of the Δ*PPE2* Δ*metE*::*hyg* double mutant with pMC*PQU*comp vector containing the full-length *PPE2-cobQ1-cobU,* restored the ability of the mutant to grow in medium supplemented with cobalt. However, the reversal of this phenotype could not be attributed to PPE2 alone; in particular, it remained possible that deletion of PPE2 (the first gene in the operon) might have had polar effects on the two downstream genes, *cobQ1* and *cobU*, both of which are predicted to catalyze key steps in *de novo* B_{12} biosynthesis (Figure 3.9). Importantly, the PPE2 deletion was not in-frame, and this might have abrogated the function of both CobQ1 and CobU, thereby disrupting cobalt- dependent biosynthesis of B_{12}. Therefore, a second complementation strategy was employed which entailed integration of a copy of the wild-type *PPE2* ORF containing 5' sequence but terminating at the native PPE2 stop codon, thus specifically excluding the *cobQ1* and *cobU* genes from the

complementation vector. Importantly, integration of the truncated complementation vector, pMC*PPE2*comp, at the *attB* site of the Δ*PPE2* Δ*metE*::*hyg* mutant (Figure 3.23B) restored the ability of the mutant to utilize cobalt for growth. This result established unequivocally the requirement for functional PPE2 in cobalt utilization in MTB.

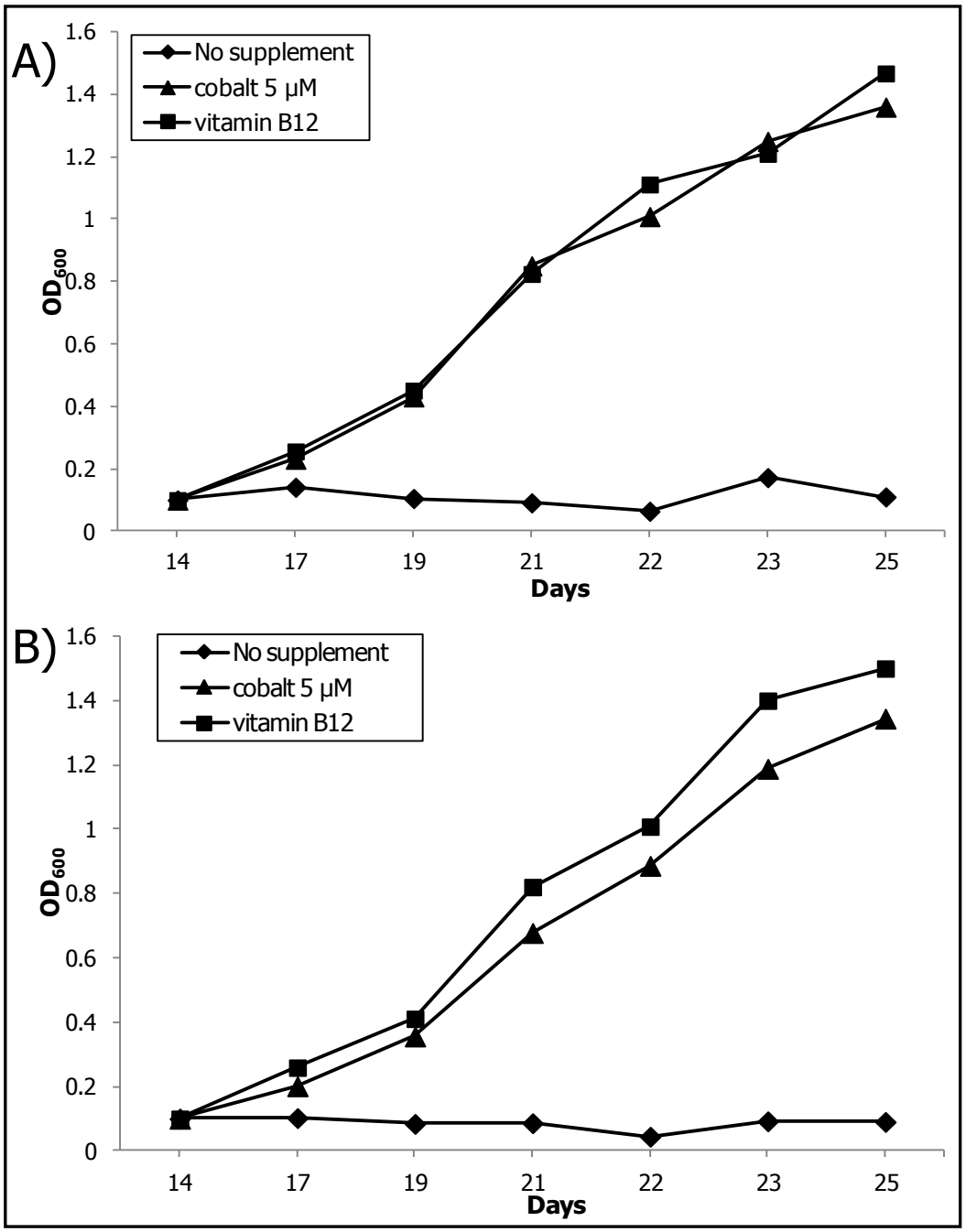

Figure 3.23: A role for *PPE2* in cobalt transport (assimilation) in MTB. Log-phase cultures of Δ*metE*::*hyg* Δ*cobUattB*::*PPE2-cobQ1-cobU* and Δ*metE*::*hyg* Δ*PPE2attB*::*PPE2* complemented strains were washed then inoculated at an OD_{600} of 0.1 into Sauton's minimal media, and grown for 8 to 14 days to allow depletion of internal B_{12}. Cultures were re-inoculated into fresh media containing the same supplement and optical densities were measured. (A) Δ*metE*::*hyg* Δ*cobUattB*::*PPE2-cobQ1-cobU* was able to grow when supplemented with cobalt (5 μM), and B) Complementation of Δ*metE*::*hyg* Δ*PPE2* with the entire *PPE2* gene restores growth when supplemented with cobalt (5 μM). Data are representative of a single experiment from three independent biological replicates.

Further support for the role of PPE2 in cobalt assimilation is provided by the observation that the Δ*PPE2* Δ*metE*::*hyg* mutant was able to utilize cobinamide for growth (Figure 3.24). In contrast, the Δ*cobU* Δ*metE*::*hyg* mutant (Figure 3.16B) was unable to utilize cobinamide, suggesting that the late stage of the pathway – that mediated by CobU – must be unaffected by the PPE2 deletion allele. That is, there appear to be no polar effects of *PPE2* deletion on the downstream B_{12} biosynthetic genes. Together, these data provide the first direct evidence of a role for a PPE protein in cation transport/assimilation; specifically, they strongly suggest a role for *PPE2* in cobalt transport/assimilation in MTB.

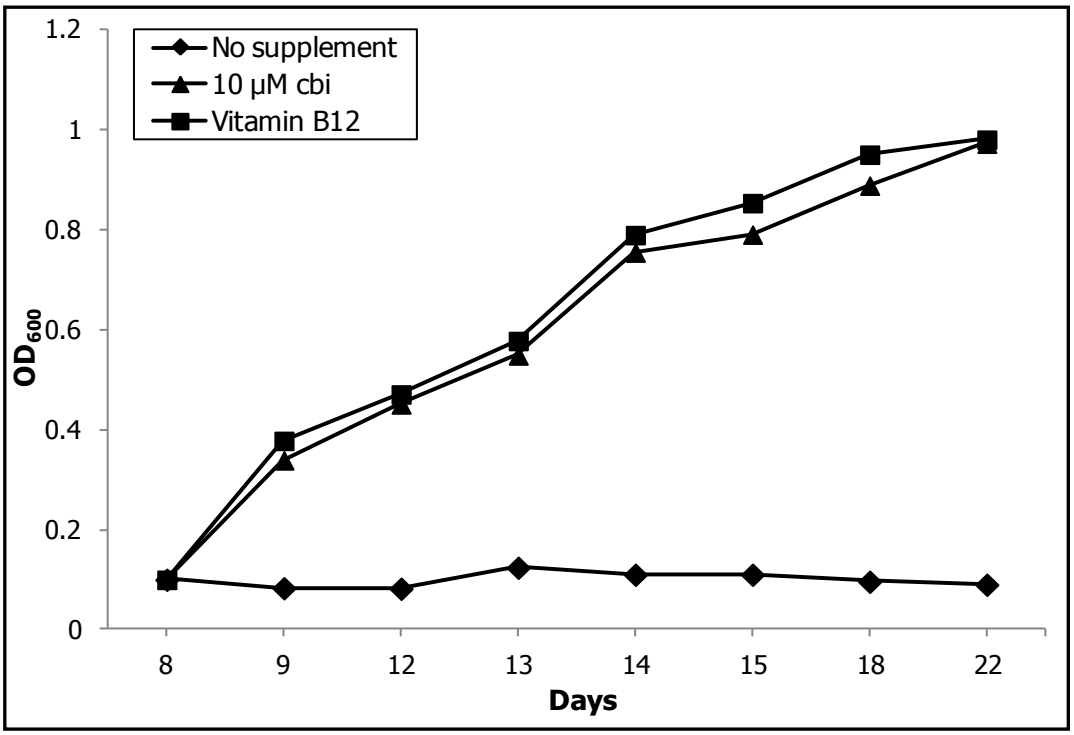

Figure 3.24: Deletion of *PPE2* does not affect growth on cobinamide in a Δ*metE*::*hyg* mutant. Log-phase cultures of Δ*PPE2* Δ*metE*::*hyg* mutant was washed then inoculated at an OD_{600} of 0.1 into Sauton's minimal media, and grown for 8 to 14 days to allow depletion of internal B_{12}. Cultures were re-inoculated into fresh media containing the same supplement(s) and optical densities were measured. Δ*PPE2* Δ*metE*::*hyg* mutant was unable to grow without supplementation (◆), but was able to grow when supplemented with B_{12} (■) and 10 µM cobinamide (▲). Data are representative of a single experiment from three independent biological replicates. Abbreviations: cbi-cobinamide.

4. Discussion

MTB possesses a near-complete B_{12} biosynthetic pathway (as discussed in section 1.6.3), as well as three B_{12}-dependent enzymes (Dawes *et al.*, 2003; Warner *et al.*, 2007; Savvi *et al.*, 2008). However, previous work in the MMRU suggested that MTB does not synthesize B_{12} *de novo* but does appear to transport the vitamin despite the lack of a candidate B_{12}-specific transporter (Warner *et al.*, 2007; Savvi *et al.*, 2008). This study investigated the functionality of the B_{12} biosynthetic pathway in MTB utilizing a genetic approach. In addition to vitamin B_{12} (CNCbl), the Δ*metE*::*hyg* mutant could be supplemented with cobinamide or cobalt, thereby providing strong evidence for *de novo* B_{12} biosynthesis in MTB. Deletion of *cobK*, whose product is required in the early (*CobI*) phase of the B_{12} biosynthetic pathway (Figure 3.9), eliminated the ability of the resulting Δ*cobK* Δ*metE*::*hyg* double mutant to utilize cobalt - but not cobinamide - for growth. In contrast, deletion of the later (*CobII*) stage enzyme, CobU, in the Δ*metE*::*hyg* background, was associated with the inability to utilize either cobalt or cobinamide for growth. Of particular note was the observation that MTB requires full-length *PPE2* in order to assimilate cobalt for growth. PPE2 is a member of the PPW subfamily of PPE proteins; although their function remains enigmatic, the results of this study provide some insight into a role for PPE proteins in the acquisition and assimilation of trace metals in MTB pathogenesis.

This project also explored the possibility that MTB synthesizes an alternate B_{12} form, pseudo-B_{12}. While the data suggest differential growth in the Δ*metE*::*hyg* mutant with increasing adenine concentrations, these assays did not allow the synthesis of pseudo-B_{12} to be established unequivocally. This is likely to be the case because the MTB mutants applied in these experiments all retain the ability to generate the α-axial ligand, DMB.

Moreover, the ability of the Δ*Rv0306* Δ*metE*::*hyg* double mutant to replicate in media supplemented with cobinamide alone again highlighted the need to determine pseudo-B_{12} levels in MTB directly utilizing alternative, biochemical techniques.

4.1 MTB does not synthesize B_{12} under standard *in vitro* conditions

MTB does not possess homologues of all genes required for vitamin B_{12} biosynthesis (discussed in section 1.6.3). Despite similar gaps in their respective B_{12} biosynthetic pathways (discussed in section 1.6.2), earlier studies utilizing microbiological assays have reported *de novo* B_{12} biosynthesis in several other mycobacterial species including *M. bovis*, *M. phlei*, MSM and *M. fortuitum*, even when grown in Sauton's minimal medium (Hendlin and Ruger, 1950; Karasseva *et al.*, 1977; Kamikubo *et al.*, 1978). An earlier study detected trace amounts of B_{12} in the culture filtrate of H37Rv (Aithal and Sirsi, 1964), however this does not necessarily indicate *de novo* B_{12} biosynthesis. In addition, previous studies in the MMRU laboratory were unable to demonstrate *de novo* B_{12} synthesis in MTB under standard *in vitro* conditions (Warner *et al.*, 2007; Savvi *et al.*, 2008). In particular, the inability of the MTB *metE* knockout to grow without exogenous B_{12} supplementation (Warner *et al.*, 2007) confirmed the *in vitro* essentiality of this gene inferred from genome-wide transposon mutagenesis (Sassetti *et al.*, 2003). Similarly, chemical inhibition of the methylcitrate cycle renders MTB unable to grow in propionate-containing media, thereby indicating that the B_{12} requirements of the methylmalonyl-CoA mutase are not met under standard *in vitro* conditions (Savvi *et al.*, 2008). These results suggest that insufficient (or no) B_{12} is produced by MTB to satisfy the cofactor requirements of these B_{12}-dependent pathways. Quantitative assays of non-industrial bacterial

B_{12} production indicate yields of between 0.2-0.8 µM (Saunders *et al.*, 1952; Musílková, 1961; Kamikubo *et al.*, 1978), which is similar to the levels of intracellular B_{12} assayed in *M. bovis* and *M. phlei* grown in Sauton's medium (Karasseva *et al.*, 1977). In turn, the failure to detect B_{12} production by MTB *in vitro* suggested that additional factors(s) might be required to stimulate (or increase) B_{12} production under standard laboratory conditions.

4.1.1 Cobalt is a limiting factor in B_{12} biosynthesis in MTB

The amount of B_{12} produced by a bacterium can be influenced by a number of factors, including the nature and quantity of the available carbon and nitrogen sources, pH, aeration, and temperature (Halbrook *et al.*, 1950; Kamikubo *et al.*, 1978; Riaz *et al.*, 2007). In this study, it was demonstrated that the MTB Δ*metE*::*hyg* mutant - which is a strict B_{12} auxotroph - can grow aerobically in liquid medium supplemented with cobalt (Figure 3.15B). This suggests that MTB has retained the ability to synthesize B_{12} provided that cobalt is not limiting. Hendlin and Ruger (1950) first reported that the availability of cobalt is among the factors that increase B_{12} production in microorganisms. In fact, in some enteric bacteria, biosynthesis is enabled (Lawrence and Roth, 1995) and even enhanced by the addition of this metal ion (Cauthen *et al.*, 1966). Interestingly, B_{12} synthesizing mycobacteria including *M. bovis* and *M. phlei* also show increased production of B_{12} upon the addition of cobalt (Karasseva *et al.*, 1977). However, the addition of cobalt can be inhibitory to growth at certain concentrations; for example, in the case of *M. bovis* which requires a lower concentration of cobalt compared to *M. phlei*. Similarly, the MTB Δ*metE*::*hyg* mutant was able to grow well in the presence of 5 µM cobalt (Figure 3.15B) whereas 10 µM cobalt was inhibitory (data not shown). It has been shown previously that the

provision of lower concentrations of cobalt to B_{12} synthesizing organisms results in as much as 75% of the metal being incorporated into the B_{12} cofactor; however, as the cobalt concentration is increased, utilization of exogenous cobalt decreases sharply (Smith *et al.*, 1952; Perlman and O'Brien, 1954). This is consistent with the fact that, while cobalt is necessary for the synthesis of B_{12} in bacteria, it is extremely toxic in excess.

It was notable that the ability of the Δ*metE*::*hyg* mutant to utilize exogenous cobalt appeared to be specific to experiments carried out in liquid media. When this mutant was grown on 7H10/OADC plates supplemented with 5 μM cobalt, it was unable to grow (Figure 3.19). A range of serially-increasing cobalt concentrations between 5 μM and 500 μM also yielded no growth (data not shown). Even when plates were supplemented with very high concentrations of cobalt – up to 10 mM - no growth was observed. The inability of cobalt to complement growth of the Δ*metE*::*hyg* mutant on solid medium is surprising, yet analogous to the ability of methionine to supplement growth of the same mutant in liquid but not on solid medium (Warner, 2006). These observations might point to an alternate bacillary physiological state that prevails in the different growth media. Alternatively, this result might indicate that PPE2-dependent assimilation of cobalt is inhibited on solid substrates.

4.1.2 Precursor-mediated growth of MTB B_{12} auxotrophs – is the B_{12} biosynthetic pathway functional?

Cobalt insertion is predicted to occur during late-stage B_{12} biosynthesis in MTB (Figure 3.9), which is consistent with aerobic pathways. Evidence supporting the ability of MTB to use exogenously supplied cobalt to synthesize B_{12} was provided in experiments utilizing specific deletion

mutants containing disruptions at certain stages of the B_{12} biosynthetic pathway, either before (*cobK*) or after (*cobU*) the predicted cobalt insertion step (Figure 3.16). The early stage enzyme, CobK, is predicted to occur in *CobI* of the pathway, and catalyzes the NADPH-dependent reduction of precorrin-6x to precorrin-6y (Blanche *et al.*, 1992b; Raux *et al.*, 1996). The *CobI* part of the B_{12} biosynthetic pathway – which involves the synthesis of the corrin macromolecule (Figure 3.9) – is absent in *E. coli*; therefore, this bacterium is unable to synthesize the corrin ring but, if provided with cobinamide, is able to synthesize and attach the α-ligand to form AdoCbl (Raux *et al.*, 1996). Similarly, deletion of *cobK* in MTB Δ*metE*::*hyg* eliminated the ability of the double mutant to grow in liquid medium supplemented with cobalt (Figure 3.16A). This is presumably because of incomplete synthesis of the corrin ring and, therefore, the inability to produce adenosylcobinamide, the precursor to AdoCbl. However, the Δ*cobK* Δ*metE*::*hyg* double mutant could still be supplemented with B_{12}, as well as the late stage precursor, cobinamide (Figure 3.16A). Since the late stage of the pathway was intact in this mutant, this result suggested that MTB has retained the ability to convert exogenously supplied cobinamide to AdoCbl, first through adenosylation to adenosylcobinamide and then by attachment of endogenously produced DMB.

Orthologues of CobK appear to fall into two distinct groups based on whether the enzymes are part of an aerobic or anaerobic B_{12} biosynthetic pathway; sequence alignments have identified several key residues that are conserved within these groups (Shearer *et al.*, 1999). It has been hypothesized (Scott, 1994) that the sequence differences might reflect the need to bind to different substrates: anaerobic CobK enzymes act on cobalt-containing intermediates whereas, in the aerobic pathway, cobalt is inserted later. An example of this selectivity was provided by the demonstration that *P. denitrifcians* CobI – which methylates precorrin-2 to

precorrin-3 - was unable to replace the *S. typhimurium* equivalent (*cbiL*), owing to the fact that the true substrate of CbiL is cobalt-precorrin-2 which is not recognised by CobI (Raux *et al.*, 1996). MTB CobK possesses key residues that are conserved in precorrin-6x reductases from aerobic or facultatively aerobic bacteria, including *P. denitrificans* and *Rhodobacter capsulatus* (Shearer *et al.*, 1999). Given the inferred specificity of these enzymes, the ability of the Δ*cobK* Δ*metE*::*hyg* double mutant to be supplemented with precorrin-6y versus cobalt-precorrin-6y would confirm whether cobalt was being inserted at early or late stage in the pathway in MTB. However, this question could not be pursued as neither intermediate is commercially available.

The late stage of the pathway was disrupted by deletion of *cobU*, a gene encoding a multifunctional enzyme of only 174 amino acids which facilitates two distinct transformations. CobU possesses kinase activity to phosphorylate adenosylcobinamide to adenosylcobinamide-phosphate, and then guanylylates adenosylcobinamide-phosphate to from adenosylcobinamide-GDP, which is in turn converted to AdoCbl by CobS (Thompson *et al.*, 1998; Thompson *et al.*, 1999). In *S. enterica*, *cobU* mutants were unable to utilize cobinamide but were able to convert cyanocobinamide-GDP to AdoCbl (O'Toole *et al.*, 1993). Consistent with this proposed function, deletion of *cobU* in the MTB Δ*metE*::*hyg* background resulted in the inability of the Δ*cobU* Δ*metE*::*hyg* double mutant to grow when supplemented with either cobalt or cobinamide (Figure 3.16B). CobS utilizes adenosylcobinamide-GDP and α-ribazole to form AdoCbl and, in *S. enterica* mutants of *cobS* were shown to be rescued with B_{12} only (O'Toole *et al.*, 1993). The MTB Δ*cobU* Δ*metE*::*hyg* double mutant was similarly able to utilize CNCbl only, strongly suggesting that disruption of the dual function CobU results in the accumulation of adenosylcobinamide, which is not the cognate substrate of CobS, and so terminates B_{12} biosynthesis prematurely.

The experiments reported here also confirmed that the active form of the cofactor synthesized from cobinamide supplementation can be utilized to support functionality of another B_{12}-dependent enzyme, the methylmalonyl-CoA mutase encoded by *mutAB*. That is, 3-NP-dependent growth inhibition of MTB on fatty acids can be alleviated when supplemented with cobinamide (Figure 3.7), owing to the enabling of MutAB function. It is also important to note that, while cobalt supplementation rescued growth of the *metE* mutant on minimal medium (Figure 3.15B) – a reaction that depends on MeCbl - this metal ion was not able to support the function of MutAB (data not shown), an AdoCbl-dependent enzyme. The reason for this discrepancy is unclear. However, Karasseva *et al.* (1977) noted that, in *M. phlei*, B_{12} production was the highest on the 30th day and intracellular cobalt levels were highest on the 23rd day of growth post inoculation, an observation which led the authors to suggest that sufficient cobalt must be accumulated first for optimal yields of B_{12} to be produced. The results reported here suggest the possibility that the simultaneous requirement for *de novo* B_{12} production against a background of accumulating propionate toxicity might exceed bacillary capacity for B_{12} biosynthesis. This interpretation is speculative, however, and will require further investigation utilizing a Δ*prpDC* mutant in order to minimize the potentially confounding effects associated with chemical inhibition of the methylcitrate cycle by 3-NP.

The ability of MTB to utilize cobalt (MetE) and cobinamide (MetE and MutAB) to support the B_{12}-dependent enzymes suggests that the active cofactor form is synthesized *de novo*. This in turn implies that the *CobI*, *CobII*, and *CobIII* stages of the B_{12} biosynthetic pathway are functional. That is, MTB is able to synthesize DMB (α-ligand) from a flavin precursor (Campbell *et al.*, 2006; Taga *et al.*, 2007) and then covalently link DMB to either endogenous (if cobalt is supplied) or exogenous cobinamide, plus a phosphoribosyl group, to produce B_{12} (Figure 3.9). Together, the results

generated here suggest that the previous inability to detect vitamin B_{12} biosynthesis in the *metH*-dependent phenotypic assay could have resulted from cobalt limitation in standard laboratory media. However, it is important to reiterate that the *de novo* B_{12} biosynthetic ability is inferred from genetic analyses: owing to the technical complexities associated with B_{12} extraction and detection, direct (biochemical) evidence of the active cofactor has yet to be obtained.

4.1.3 Why does MSM not require cobalt supplementation to synthesize B_{12}?

The ability of the Δ*metE*::*hyg* mutant of MSM to grow without any supplementation suggests that MSM is able to synthesize B_{12} *in vitro* (Figure 3.1). In contrast, growth of the corresponding MTB Δ*metE*::*hyg* requires that the same medium must be supplemented with 5 μM cobalt (Figure 3.15B). In a previous study, an elemental analysis of Sauton's minimal medium by plasma mass spectrometry revealed a cobalt concentration of 5 nM (Campbell *et al.*, 2007). It is likely, therefore, that traces of the metal ion were present in the medium utilized in this study. Analysis of the MSM genome indicates the presence of at least five putative cobalt transport systems (K. Gopinath, personal communication), three of which are homologues of well-defined cobalt transporters including CbiMQO, CbtAB and CorA (Nelson and Kennedy, 1971; Roth *et al.*, 1993; Rodionov *et al.*, 2003). By comparison, MTB appears to possess a homologue of CorA only (Agranoff and Krishna, 2004), as well as two other predicted cobalt transporters, Rv2325c-Rv2326c and PPE2 (Rodionov *et al.*, 2003; Campbell *et al.*, 2007; Rodionov *et al.*, 2009). This suggests that the different lifestyles of the non-pathogenic environmental saprophyte and the obligate human pathogen might require different cobalt scavenging capacity. In turn, the abundance of cobalt transporters

in MSM compared to MTB might ensure that MSM is better able to acquire cobalt *in vitro* and, therefore, does not require supplementation.

Table 4.1: Growth characteristics in liquid media of MTB mutants utilized in this study

Strain	**Liquid medium**	**No supplement**	**B_{12}**	**Co**	**cbi**	**cbi plus DMB**	**cbi plus AD**
H37Rv	S	✓	✓	✓	✓	✓	✓
H37Rv	VL+3-NP	×	✓	×	✓	✓	✓
Δ*PPE2*	S	✓	✓	✓	✓	✓	✓
Δ*PPE2*	VL+3-NP	×	✓	×	✓	✓	✓
Δ*metE*::*hyg*	S	×	✓	✓	✓	✓	✓
Δ*cobK* Δ*metE*::*hyg*	S	×	✓	×	✓	✓	✓
Δ*PPE2* Δ*metE*::*hyg*	S	×	✓	×	✓	✓	✓
Δ*cobU* Δ*metE*::*hyg*	S	×	✓	×	×	×	×
Δ*metE*::*hyg* Δ*PPE2attB*::*PPE2*	S	×	✓	✓	✓	✓	✓
Δ*metE*::*hyg* Δ*PPE2 attB*::*PPE2-cobQ1-cobU*	S	×	✓	✓	✓	✓	✓
Δ*metE*::*hyg* Δ*cobU attB*::*PPE2-cobQ1-cobU*	S	×	✓	✓	✓	✓	✓
Δ*Rv0306*	S	×	✓	✓	✓	✓	✓
Rv0306^{D32NS167G}Δ*metE*::*hyg*	S	×	✓	✓	✓	✓	✓

Key: **S** – Sauton's minimal medium
VL – valerate
Co – 5μM cobalt
cbi – cobinamide
(✓) – indicates growth
(×) – indicates no growth
3-NP – 3-nitroproprionate
DMB – 5, 6- dimethylbenzamidazole
AD - adenine

4.2 PPE2 is required for cobalt transport/assimilation in MTB

Riboswitches are mRNA elements (Vitreschak *et al.*, 2003) that serve as ligand-responsive genetic controls to modulate the expression of specific genes in response to changing concentrations of metabolites (Nahvi *et al.*, 2004). These metabolites include amino acids such as lysine and S-adenosylmethionine (Rodionov *et al.*, 2003; Winkler *et al.*, 2003), and metal ions such as magnesium (Dann III *et al.*, 2007) and molybdenum cofactor (Regulski *et al.*, 2008b). In general, B_{12}-riboswitches occur upstream of genes falling into three broad categories: those involved in transport of B_{12} or cobalt (Richter-Dahlfors and Andersson, 1992; Cheng *et al.*, 2011), those required for B_{12} biosynthesis (Lundrigan *et al.*, 1991) and, finally, those encoding B_{12}-dependent enzymes which possess corresponding B_{12}-independent alternatives (Borovok *et al.*, 2006). In all cases, these riboswitches regulate expression of the associated downstream gene(s) in response to B_{12} (Borovok *et al.*, 2006; Warner *et al.*, 2007). In prior work from the MMRU, Warner *et al.* (2007) identified a riboswitch upstream of MTB *metE*, and demonstrated its functionality. This was the first riboswitch confirmed in MTB and is one of only two B_{12}-riboswitches that have been identified the genome of this organism. The second B_{12}-riboswitch is located immediately upstream of *PPE2*, which is the first gene in a putative three-gene operon comprising two B_{12} biosynthetic genes, *cobQ1* and *cobU*. Based on its association with a B_{12}-riboswitch, its genomic context, and the prediction that the PPE2 protein possesses seven trans-membrane segments, Rodionov *et al.* (2003) predicted that *PPE2* encodes a putative cobalt transporter. In *S. meliloti*, the *smb20056* gene – which is similarly associated with an upstream B_{12}-riboswitch – was predicted to encode a *btuFCD*-type B_{12} transporter together with the downstream genes *smb20057* and *smb20058* (Vitreschak *et al.*, 2003). However, it was recently shown that these genes in fact encode a cobalt transporter (Cheng *et al.*, 2011). In the present

study, the paucity of information available regarding the function of PPE genes, as well as the absence of a clear B_{12} transport candidate in the MTB genome (Rodionov *et al.*, 2003; Warner *et al.*, 2007), motivated our investigation of both possibilities: that is, that PPE2 might function in B_{12} and/or cobalt transport.

A genetic approach was used to confirm that PPE2 is not involved in B_{12} transport in MTB. Firstly, transcriptional analysis demonstrated classic down-regulation of *metE* expression in a Δ*PPE2* mutant in response to B_{12} treatment (Figure 3.20), thereby suggesting the intracellular accumulation of exogenously supplied vitamin B_{12} even in a strain lacking functional PPE2. Secondly, the transport of B_{12} *in vitro* remained unaffected in a Δ*PPE2* Δ*metE*::*hyg* double mutant, as inferred from the ability of the mutant to grow in B_{12}-supplemented media (Figure 3.24). Finally, in an assay that utilized chemical inhibition of ICL when MTB was grown on fatty acids, growth inhibition of the Δ*PPE2* mutant was shown to be alleviated upon B_{12} addition in a manner that was identical to that observed for wild-type H37Rv (Figure 3.21A & B). Together, these data eliminated a role for PPE2 in B_{12} transport. Additionally, PPE2 shares no homology with experimentally characterized B_{12}-specific transporters such as the *E. coli* BtuBFCD transporter. Furthermore, recent investigations in the MMRU (K. Gopinath *et al.*, manuscript in preparation) have identified a candidate protein which, at least *in vitro*, appears to function as the sole B_{12} transporter in MTB.

In contrast to the parental Δ*metE*::*hyg* strain (Figure 3.15B), the Δ*PPE2* Δ*metE*::*hyg* double mutant was unable to grow in minimal medium supplemented with cobalt (Figure 3.22B). Bioinformatic analyses predict the existence of three putative cobalt transporters in the MTB genome, encoded by *corA*, *Rv2325c-RV2326c* and *PPE2*. For this reason, deletion of *PPE2* in the Δ*metE*::*hyg* mutant might not be expected to affect the ability

of the auxotroph to assimilate cobalt for B_{12} synthesis, as there are two possible alternate transporters available. Moreover, a diversity of non-specific metal uptake systems are present in MTB, four of which are predicted to transport cobalt ions in addition to other metals (Agranoff and Krishna, 2004). In *S. meliloti*, deletion of the cobalt transport system encoded by *cbtJKL* resulted in decreased accumulation of cobalt relative to the wild-type strain (Cheng *et al.*, 2011). This result suggested that non-specific transporters - for example CorA, which is predicted to transport magnesium, cobalt and iron (Niegowski and Eshaghi, 2007; Zhang and Gladyshev, 2009) - are able to take up cobalt but are probably not physiologically relevant as they possess a lower affinity for the metal ion.

Cobalt uptake proteins in microorganisms usually exhibit shared architecture (Rodionov *et al.*, 2003; Siche *et al.*, 2010), which facilitates their identification in the genome. For example, Rv2325c, which is proposed to be an energy-coupling transporter (ECF) (Rodionov *et al.*, 2009), has been shown to possess a CbiQ domain. Furthermore, Rv2325c appears to be in an operon with Rv2326c, which itself has a CbiO domain (K. Gopinath; personal communication), and so is suggestive of a potential role in cobalt or even B_{12} transport. However, a similar analysis of PPE2 does not reveal the presence of conserved structural domains characteristic of known or predicted cobalt transporters.

Despite the lack of any conserved structural architecture for PPE2 as a predicted cobalt transporter, the inability of cobalt to restore growth of the Δ*PPE2* Δ*metE*::*hyg* double mutant was demonstrated in this study (Figure 3.15B & 3.22B). In addition, the phenotypes associated with deletion of *cobK* or *cobU* in the Δ*metE*::*hyg* mutant background (Figure 3.16), strongly suggest that MTB incorporates exogenous cobalt into B_{12}. The genetic data presented in this study also indicate that PPE2 is critical to this process. Therefore, one might speculate that PPE2 plays an integral

role in regulating the amount of cobalt in the bacterial cell or that it is required to scavenge cobalt and facilitate its delivery to cobalt uptake systems. In the latter option, proteins that facilitate transfer or transport of metal ions to the required site usually possess N-terminal or C-terminal metal binding motifs (Okamoto and Eltis, 2011) which have a high affinity for the metal ion and, in this case, would act as a cobalt store or cobalt trap (Raux *et al.*, 2003). Binding of cobalt is predicted to occur by a histidine-rich motif (Rodionov *et al.*, 2003) which, in the cobaltochelatase encoded by CbiK in *Bacillus megaterium*, is present in the C-terminal region; deletion of this region results in decreased affinity for the metal ion (Raux *et al.*, 2003). However, in the PPE2 protein sequence, no histidine-rich N-terminal or C-terminal motif could be identified. Additional signatures for cobalt binding sites have been identified (Thilakaraj *et al.*, 2007), yet none of these appears to be present in PPE2. Interestingly, sequence-based analysis of PPE2 utilizing the *Support Vector Machine* server (Lin *et al.*, 2006) indicates the highest probability of binding zinc, despite the absence in PPE2 of cysteine residues that are typical of zinc binding proteins (A. Zawaira; personal communication).

The arrangement of a *PPE2* gene together with an upstream B_{12} riboswitch is conserved among the slow-growing mycobacterial species (MTB, *M. ulcerans*, *M. marinum M. avium, M. paratuberculosis* and *M. leprae*), irrespective of genomic context, but is absent in the fast-growing MSM. PPE2 has been identified in the cell membrane fraction of MTB (Mawuenyega *et al.*, 2005), although the gene does not appear to be differentially expressed in macrophages or in mice infected with MTB (Schnappinger *et al.*, 2003; Talaat *et al.*, 2004; Tailleux *et al.*, 2008). Microarray studies have indicated that *PPE2* is down-regulated after 24 h of starvation and induced after 30 minutes in standing cultures of MTB (Betts *et al.*, 2002; Kendall *et al.*, 2004). It is notable, however, that global responses of MTB to other metals such as copper, zinc, and iron do

not indicate any significant up- or down-regulation of *PPE2* in the presence of toxic or physiological concentrations of these metals (Rodriguez *et al.*, 2002; Maciag *et al.*, 2007; Ward *et al.*, 2008). Therefore, aside from the prediction of Rodionov *et al.* (2003) and the data described in this study, there appears to be no other evidence to support a role for PPE2 in cobalt transport/assimilation.

4.2.1 What is the function of the PE/PPE protein family?

The size of the PE/PPE family suggests the possibility of functional redundancy, although this has not been formally demonstrated. It is unclear, therefore, why PPE2 should be specifically required for cobalt/transport assimilation in MTB. That is, why is the phenotype of the PPE2 knockout so profound? An analysis by van Pittius *et al.* (2006) indicates that PPE3 (Rv0280) of the PPW subfamily is very closely related to PPE2 and, interestingly, PPE3 has been shown to be up-regulated in response to zinc (Maciag *et al.*, 2007). Another member of the PPW subfamily, PPE37, was up-regulated under iron-deficient conditions and was suggested to be a siderophore-type protein involved in iron uptake (Rodriguez *et al.*, 1999; Rodriguez *et al.*, 2002). More recently, this protein has also been implicated in host immune evasion by interfering with proinflammatory cytokines (Daim *et al.*, 2011). Other members of the PPW family include PPE46, whose inactivation was shown to attenuate TB in mice (Camacho *et al.*, 1999) and was shown to be essential *in vitro* (Sassetti *et al.*, 2003); PPE47, which was up-regulated at least eightfold in human brain microvascular endothelial-cells and has been suggested to be crucial for intracellular survival and endothelial-cell invasion (Jain *et al.*, 2006); and PPE4 and PPE20 which are down-regulated during nutrient starvation (Betts *et al.*, 2002). Thus far, however, studies of proteins belonging to this subfamily do not allude to any specific function in MTB.

Although the exact function(s) of the PE/PPEs is not known, several members of this family have been proposed to be virulence factors (Ramakrishnan *et al.*, 2000; Brennan *et al.*, 2001; Li *et al.*, 2005), and some have even been shown to elicit strong immune responses in animals and humans (Dillon *et al.*, 1999; Skeiky *et al.*, 2000; Okkels *et al.*, 2003; Singh *et al.*, 2005). In a recent study, the SVP subfamily member, PPE17, was shown to augment transcription of HIV-1 by interacting with TLR2 receptors which activated signalling pathways favourable to HIV-1 replication (Bhat *et al.*, 2012). It is interesting, however, that another SVP subfamily protein - PPE18, whose interaction with TLR2 receptors was demonstrated to inhibit host immune responses and thus promote replication of MTB (Nair *et al.*, 2009) - was unable to augment HIV-1 replication by this pathway (Bhat *et al.*, 2012). These studies add to the accumulating evidence which suggests specific, non-redundant roles for the PPE proteins in modulating or altering the immune response by interacting with host immune components. As such, some PE/PPEs appear to be ideally positioned to interact with the host immune system as they have been shown to be cell-wall-associated (Banu *et al.*, 2002; Okkels *et al.*, 2003; Le Moigne *et al.*, 2005) or even partially exposed on the cell surface (Brennan *et al.*, 2001; Sampson, 2011). In turn, this suggests the possibility that they might be secreted by the bacterium into the host cell. Secretion of selected PE/PPE proteins occurs in an ESX-5 dependent manner in *M. marinum* (Abdallah *et al.*, 2006; Abdallah *et al.*, 2008; Abdallah *et al.*, 2009) and, more recently, this system was shown to be important for cell wall integrity and virulence in MTB (Bottai *et al.*, 2012). Notably, the ESX-3 cluster appears to be regulated by iron and zinc in MTB (Maciag *et al.*, 2007), suggesting involvement of this system in maintaining homeostasis of these metal ions. Although the PE/PPEs have largely been implicated in antigenic variation and disease pathogenesis (Sampson, 2011), some members of this family were demonstrated to have fibronectin-binding properties (Espitia *et al.*, 1999). There are even

reports of enzymatic functions, such as the phosphoglycerate mutase activity associated with PE_PGRS11 (Chaturvedi *et al.*, 2010).

It is tempting to speculate that the various subfamilies might be separated according to a broad function – that is, can a role in metabolite/metal ion homeostasis be generally ascribed to members of the PPW subfamily, whereas SVP subfamily members might be broadly involved in immune modulation? This might be too simplistic, and further analysis is required. In summary, therefore, it appears that proteins belonging to this intriguing family appear to have diverse functions which are not readily predicted by bioinformatic analyses of intrinsic properties including sequence or structural features, or more global characteristics such as genomic context or transcriptional regulation.

4.3 A role for B_{12} or cobalt in MTB pathogenesis?

In humans, only two enzymes require B_{12} for activity: methylmalonyl-CoA mutase and methionine synthase (Martens *et al.*, 2002). Vitamin B_{12} deficiency owing to low intake or malabsorption has been shown to cause pernicious anaemia and neurological dysfunction, and has also been linked with cardiovascular disease (McCaddon *et al.*, 1994; Pancharuniti *et al.*, 1994; Clarke *et al.*, 1998; Choi and Mason, 2002). The daily required intake of vitamin B_{12} in humans is estimated at 0.9-2.4 µg. Since this essential micronutrient is synthesized by selected microorganisms only, it is acquired through the consumption of foods like milk, eggs, fish and meat (Watanabe, 2007). As a result, elaborate and complex mechanisms of absorption, transport, and cellular uptake of B_{12} exist in humans to ensure that the cofactor is available in the sub-cellular locations in which the B_{12}-dependent enzymes operate.

Following ingestion, food-bound B_{12} is released in the stomach by the action of hydrochloric acid, and then immediately bound by haptocorrin (HC), the chaperone which protects it from chemical denaturation in the harsh acidic environment (Banerjee, 2006). Bound B_{12} is transported to the duodenum where the B_{12} is liberated from HC by pancreatic protease, and immediately captured by another chaperone, intrinsic factor (IF), in an IF-Cbl complex (Banerjee *et al.*, 2009). The IF-Cbl complex is subsequently transported from the duodenum and, after degradation of IF, B_{12} is taken up in the bloodstream, where it can attach to either HC or transcobalamin II (TCII) (Morkbak *et al.*, 2006). Although the bulk of B_{12} is bound to HC (~80%), TC bound B_{12} (TCII-Cbl) facilitates uptake into cells (Hall, 1977; Morkbak *et al.*, 2006). From the bloodstream, the TCII-B_{12} complex is taken up into the cell via receptor-mediated endocytosis into the lysosome (Youngdahl-Turner *et al.*, 1978). The acidic environment within the endocytic lysosome digests the TCII-Cbl complex, and releases the B_{12} into the cytoplasm where it becomes available for the B_{12}-dependent methionine synthase and mitochondrial methylmalonyl-CoA mutase (Youngdahl-Turner *et al.*, 1978; Youngdahl-Turner *et al.*, 1979; Padovani *et al.*, 2008).

As described above, the passage of B_{12} through the body via multiple chaperones terminates with lysosome-mediated delivery of the cofactor to target cells. It is tempting, therefore, to speculate that B_{12} might effectively be "delivered to the doorstep" (R. Banerjee, personal communication) of MTB in the context of intracellular infection. In this regard, it is instructive to recall that the methylcitrate pathway is dispensable *in vivo* in the mouse model of TB infection, an observation which suggests the functioning of the B_{12}-dependent methylmalonyl pathway as an alternative route for propionyl-CoA detoxification (Muñoz Elías *et al.*, 2006; Savvi *et al.*, 2008). In turn, this points to the potential availability of vitamin B_{12} during infection – at least in mice – whether

synthesized endogenously or acquired from the host. The amount of B_{12} available *in vivo* varies with diet; for this reason, no specific intracellular B_{12} concentration has been determined, although the total amount of stored B_{12} is estimated at 2-4 mg in adults, of which 30-60% is stored in the liver (McLaren, 1981). It remains uncertain how much of this is available at the site of infection (if any) and whether MTB is able to access intracellular vitamin B_{12}. Interestingly, in certain vegetarian populations, low B_{12} levels have been associated with increased risk of TB (Chanarin and Stephenson, 1988; Strachan *et al.*, 1995; Yajnik *et al.*, 2006). Conversely, enhanced growth of MTB is observed *in vitro* upon addition of B_{12} (Savvi, 2009). While these observations might be interpreted as having conflicting implications for the role of B_{12} in pathogenesis, it must be borne in mind that B_{12} deficiency is likely to have multiple effects on host immune function. Therefore, extrapolating a result from a population with a very specific dietary bias, as well as a hallmark B_{12} deficiency, is complicated. In this regard, it would be interesting to investigate the potential association with TB susceptibility of any of the eight vitamin B_{12} complementation groups (Banerjee *et al.*, 2009) which define inherited disorders in B_{12} metabolism.

The demonstration in this study that cobalt can complement growth of a MTB B_{12} auxotroph (Figure 3.15B) similarly raises an important question pertaining to the availability of cobalt during infection. Studies on the elemental analysis of phagosomes have identified other metals (Wagner *et al.*, 2005; Wagner *et al.*, 2006), but cobalt is a trace nutrient and as such is mainly available in B_{12}. It appears, therefore, that further work is required to determine the exact availability of this metal ion to MTB *in vivo*.

4.4 Can MTB synthesize and utilize pseudo-B_{12}?

The inferred ability of MTB to adapt to multiple environments *in vivo* suggested that the bacillus might be able to utilize alternate B_{12} cofactors, including pseudo-B_{12}, in which adenine substitutes for DMB as α-axial ligand (Figure 1.1). Titrations performed with adenine and 10 μM cobinamide showed no differential growth in the MTB *metE* mutant (Figure 3.8B). However, it was noted that supplementing the medium with 5 μM cobinamide resulted in reduced growth in combination with concentrations of 0.01 μM and 0.1 μM adenine – in contrast to 5 μM cobinamide alone (Figure 3.8A). Thereafter, a slight differentiation in growth of MTB in response to 5 μM cobinamide and increasing concentrations of adenine was observed (Figure 3.8A). A similar trend was observed in the MSM adenine titrations (Figure 3.5B), where 10 μM and 25 μM adenine (plus 10 μM cobinamide) resulted in decreased growth compared to 10 μM cobinamide alone. Again, improved growth was associated with increasing concentrations of adenine and 10 μM cobinamide (Figure 3.5B). Adenine toxicity has been demonstrated in *E. coli* (Levine and Taylor, 1982), but it is unlikely that the decreased growth observed with low-dose adenine can be attributed to this effect: both MSM and MTB were able to tolerate much higher concentrations (100 μM) of adenine which resulted in improved growth of the B_{12} auxotrophs when provided in combination with cobinamide. It is possible that lowering the concentration of cobinamide further will allow better de-convolution of this phenotype, which is suggestive of a regulatory switch.

Based on the results presented here, it is only possible to conclude that supplementation with adenine and cobinamide in either MSM (Δ*cobK* Δ*metE*::*hyg*) or MTB (Δ*metE::hyg*) was only slightly suggestive of pseudo-B_{12} synthesis. Instead, it appears that an alternative biochemical approach will be required to differentiate the specific chemical species; for example,

direct detection by HPLC (Anderson *et al.*, 2008). This approach was not pursued in this project owing to the technical complexities (and associated health and safety risks) of B_{12} extraction and detection, which involves the use of cyanide. Furthermore, pseudo-B_{12} is not commercially available and extraction would therefore require isolation of the alternate cofactor from an organism that synthesizes pseudo-B_{12}. A recent study by Szterk and colleges (2012) described an alternative technique of extracting B_{12} which, although not as sensitive, does offer the possibility of qualitative assessment of the various B_{12} forms in MTB.

4.4.1. Deletion of **Rv0306** *in MTB*

The genetic approach utilized in this study to elucidate whether MTB has the capacity to synthesize pseudo-B_{12} was based on the assumption that abrogation of BluB function would eliminate the ability of the *metE* mutant to utilize cobinamide for growth in minimal medium. This approach relied heavily on the prediction that *Rv0306* encodes the MTB *bluB* homologue (Campbell *et al.*, 2006; Taga *et al.*, 2007) and, further, that MTB does not possess an alternative pathway for DMB biosynthesis. The BluB enzyme was demonstrated both experimentally (Pollich and Klug, 1995; Campbell *et al.*, 2006) and biochemically (Taga *et al.*, 2007) to be responsible for the aerobic conversion of flavin mononucleotide to DMB in other organisms. In addition to bioinformatic searches carried out in this study, multiple other studies (Rodionov *et al.*, 2003; Campbell *et al.*, 2007; Taga *et al.*, 2007) identified Rv0306 as the candidate BluB in MTB. It was notable that deletion of *Rv0306* in the Δ*metE*::*hyg* mutant background did not inhibit the ability of MTB to utilize cobalt (data not shown), thereby invalidating the previous prediction of Rv0306 as a putative cobalt reductase (Figure 3.9). It was surprising, however, that the Δ*Rv0306* Δ*metE*::*hyg* double mutant was able to grow when supplemented with

cobinamide alone (Figure 3.14), since this implied an alternative mechanism for DMB biosynthesis, or that MTB was able to counter the loss of this enzyme by producing a pseudo-B_{12} form (or forms) using an alternative, endogenous α-ligand.

This observation was, however, consistent with predictions made by Anderson and colleagues: in their paper describing the elucidation of pseudo-B_{12} biosynthesis in S. enterica, the authors postulated that the phenotypic identification of the effects of disrupted DMB synthesis was expected to be difficult owing to the fact that, in the absence of DMB, corrinoid synthesis would shift to pseudo-B_{12} production, without any impairment of growth (Anderson *et al.*, 2008).

S. enterica synthesizes pseudo-B_{12} under anaerobic conditions (Keck and Renz, 2000), and "orthodox" B_{12} under aerobic or microaerophillic conditions when provided with cobinamide (Johnson and Escalante-Semerena, 1992). *S. enterica* appears not to possess a *bluB* homologue (Keck *et al.*, 1998; Anderson *et al.*, 2008), raising the possibility that another enzyme might carry out this function, which might be present in MTB as well. *M. leprae* has undergone extensive genome reduction (Cole *et al.*, 2001) but is in possession of the late stage B_{12} biosynthetic enzymes, including CobS and CobT, excluding a putative *bluB* homologue. *M. leprae* is also thought to be an excellent adenine scavenger (Wheeler, 1987), which suggests that pseudo-B_{12} might be a relevant cofactor in different mycobacteria. As noted above, a more precise method such as the use of HPLC (Anderson *et al.*, 2008) or equivalent (Szterk *et al.*, 2012) to detect the exact chemical species utilized by different mycobacteria will be required to confirm this prediction.

4.5 Concluding remarks

Utilizing a purely genetic approach, this study has established that cobalt supplements growth of the MTB *metE* mutant, a B_{12} auxotroph. However, fundamental questions remain, most of which relate to the role of vitamin B_{12} biosynthesis in MTB pathogenesis. These include: does MTB synthesize B_{12} *in vivo*? If so, how much of cobalt is available and can MTB access this metal ion from the host? Does MTB utilize host transporters or chaperones? Alternatively, if B_{12} is acquired from the host – how much is available and under what conditions is this cofactor accessed?

Although the phenotypes associated with deletions of genes predicted to occur in early and late stages of the B_{12} biosynthetic pathway implied that cobalt was being utilized for *de novo* vitamin B_{12} synthesis, the use of radiolabelled cobalt would better aid in confirming accumulation and transport of this metal ion in MTB. This would be especially useful in the presence and absence of functional PPE2, to confirm the role of this gene in cobalt transport/assimilation. Furthermore, transcriptional analyses by microarray of MTB in response to cobalt could possibly elucidate regulation of PPE2 to this metal ion, although this is less certain based on similar transcriptional profiling of PPE genes (described in section 1.11.1). Given the inferred role of PPE2 in cobalt transport/assimilation, a recombinant form PPE2 might better elucidate cellular localization, and would offer the possibility of gaining structural insight into this unexpected function. Also, the predicted cobaltochelatase function of CysG could be explored by deleting *cysG* in the Δ*metE*::*hyg* background; this might be predicted to phenocopy the Δ*cobK* Δ*metE*::*hyg* and Δ*PPE2* Δ*metE*::*hyg* mutants if *cysG* is required for cobalt insertion in MTB. Lastly, chemical differentiation of the exact species of B_{12} synthesized when Δ*metE*::*hyg* is supplemented with cobalt, and when Δ*Rv0306* Δ*metE*::*hyg* is supplement

with cobinamide alone or in combination with adenine or DMB, will better resolve the exact nature of the cofactor synthesized by MTB.

5. Appendices

Appendix A: List of abbreviations

2TY	Tryptone Yeast broth
3-NP	3-nitropropionate
AD	Adenine
AdoCbl	Adenosylcobalamin
Amp	Ampicillin
aph	Aminoglycoside phosphotransferase, confers resistance to kanamycin
BCG	bacille Calmette-Guérin
bla	Gene conferring resistance to ampicillin
bp	Base pair
BSA	Bovine serum albumin
cbi	Cobinamide
Cbl	Cobalamin
CNCbl	Cyanocobalamin (vitamin B_{12})
CTAB	Cetyltrimethylammonium bromide
d	Day(s)
DMB	5,6-dimethylbenzamidazole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOTS	Directly Observed Therapy – Short Course
EDTA	Ethylenediaminetetraacetic acid

fM	femtomolar
g	Gravitational force
g	grams
GS	Glucose salt
h	Hour(s)
HC	Haptocorrin
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HPLC	High-performance liquid chromatography
hyg	Gene conferring resistance to hygromycin B
Hyg	Hygromycin B
ICL	Isocitrate lyase
IF	Intrinsic factor
IRIS	Immune Reconstitution Inflammatory Syndrome
Km	Kanamycin
LA	Luria-Bertani agar
lacZ	Gene encoding β-galactosidase
LB	Luria-Bertani broth
LTBI	Latent tuberculosis infection
MDR	Multidrug Resistant
MeCbl	Methylcobalamin
min	Minutes
ml	Mililitre
MTB	Mycobacterium tuberculosis
NaCl	Sodium chloride

$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NCE	Non-citrate ethanolamine
nt	Nucleotide
OAA	Oxaloacetate
OADC	Oleic acid-albumin-dextrose-catalase
OD_{600}	Optical density at 600 nanometre wavelength
OHCbl	Hydroxycobalamin
PCR	Polymerase Chain Reaction
PDIM	Phthiocerol dimycocerosate
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
RD1	Region of difference-1
RNA	Ribonucleic acid
RT	Reverse transcription/transcriptase
s	Seconds
sacB	Gene encoding levansucrase
SAM	S-adenosyl-L-methionine
sdH_2O	Sterile distilled water
SDS	Sodium dodecylsulphate
SL	Sulfolipid
Suc	Sucrose
TAG	Triacylglycerol
TB	Tuberculosis
TCA	Tricarboxylic acid

TCII	Transcobalamin II
TDR	Totally Drug Resistant
TE	Tris-EDTA
TLR	Toll-like receptor
Tris	Tris(hydroxymethyl)aminomethane
Tween	Polyoxyethylene sorbitan monooleate
v/v	Volume per volume
v/w	Weight per volume
WHO	World Health Organization
XDR	Extensively Drug Resistant
X-gal	5-bromo-4-chloro-3-indolyl-D-thiogalactopyranoside

Appendix B: Culture media

All media are made up to a final volume of 1 litre with deionised water, and sterilised by autoclaving at 121°C for 20 minutes, unless otherwise stated.

Luria-Bertani Broth (LB)

5g yeast, 10g tryptone, 10g sodium chloride

Luria-Bertani Agar (LA)

5g yeast, 10g tryptone, 10g sodium chloride, 15g agar

2TY

5g sodium chloride, 10g yeast extract, 16g tryptone

Middlebrook 7H9

2ml glycerol, 4.7g Difco™ Middlebrook 7H9 broth

Middlebrook 7H10

5ml glycerol, 19g Difco™ Middlebrook 7H10 agar

Sauton's minimal medium (pH 7.2)

4 g asparagine, 0.5 g magnesium sulphate, 2 g citric acid, 0.5 g potassium dihydrogen orthophosphate, 0.05 g ammonium ferric citrate, 48ml glycerol. Sterilised by filtration.

Valerate

4.7g DifcoTM Middlebrook 7H9 broth, 0.85g NaCl, 5g BSA, 1ml valeic acid. pH 6.8; sterilise by filtration.

NCE medium

0.2g magnesium sulphate, 10g dipotassium phosphate, 3.5g 0.2g magnesium sulphate, 10g dipotassium phosphate, 3.5g Ammonium sodium phosphate dibasic tetrahydrate, 3.9g ethanolamine hydrochloride. pH7.0; sterilise by filtration

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