A vitamin $B_{12}$ transporter in *Mycobacterium tuberculosis*

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1. Summary

Vitamin $B_{12}$-dependent enzymes function in core biochemical pathways in *Mycobacterium tuberculosis*, an obligate pathogen whose metabolism *in vivo* is poorly understood. Although *M. tuberculosis* can access vitamin $B_{12}$ *in vitro*, it is uncertain whether the organism is able to scavenge $B_{12}$ during host infection. This question is crucial to predictions of metabolic function, but its resolution is complicated by the absence in the *M. tuberculosis* genome of a direct homologue of BtuFCD, the only bacterial $B_{12}$ transport system described to date. We applied genome-wide transposon mutagenesis to identify *M. tuberculosis* mutants defective in their ability to use exogenous $B_{12}$. A small proportion of these mapped to *Rv1314c*, identifying the putative PduO-type ATP : corrinoid adenosyltransferase as essential for $B_{12}$ assimilation. Most notably, however, insertions in *Rv1819c* dominated the mutant pool, revealing an unexpected function in $B_{12}$ acquisition for an ATP-binding cassette (ABC)-type protein previously investigated as the mycobacterial BacA homologue. Moreover, targeted deletion of *Rv1819c* eliminated the ability of *M. tuberculosis* to transport $B_{12}$ and related corrinoids *in vitro*. Our results establish an alternative to the canonical BtuCD-type system for $B_{12}$ uptake in *M. tuberculosis*, and elucidate a role in $B_{12}$ metabolism for an ABC protein implicated in chronic mycobacterial infection.

2. Introduction

The genome of *Mycobacterium tuberculosis*, obligate human pathogen and causative agent of tuberculosis, encodes three $B_{12}$-dependent enzymes. Previous work in our laboratory has established that both the methylmalonyl-coenzyme A (CoA) mutase, MutAB [1], and the *metH*-encoded methionine synthase [2] are functional, and require $B_{12}$ for activity. *Mycobacterium tuberculosis* also possesses a predicted pathway for $B_{12}$ biosynthesis [3], but appears not to produce the cofactor *in vitro* [1,2] or in macrophages [4]. Nevertheless, the bacillus can use exogenous vitamin $B_{12}$ and encodes a $B_{12}$-responsive riboswitch that suppresses transcription of the alternative, $B_{12}$-independent methionine synthase, *metE*, in $B_{12}$-replete conditions [2]. These observations imply a role for the cofactor...
in *M. tuberculosis* pathogenesis. However, it is uncertain whether B12 is available during infection, and which mycobacterial genes are required for its uptake and assimilation.

Vitamin B12 and B12 derivatives are members of the cobalamin group of corrinoid macrocycles [5]. Cobalamins are structurally complex, comprising a defining tetrapyrrole framework with a centrally chelated cobalt ion held in place by a lower axial base, dimethylbenzimidazole and an upper ligand that determines the cofactor form (figure 1). The cyan group in vitamin B12 (cyanocobalamin, CNCbl) must be replaced by deoxyadenosine and methyl ligands, respectively, during conversion to the biologically active cofactors: adenosylcobalamin (AdoCbl or coenzyme B12), which is required by methylmalonyl-CoA mutase, and methylcobalamin (MeCbl), which serves as an intermediary in the synthesis of methionine from homocysteine and methyltetrahydrofolate [6]. The reactivity of B12 cofactors derives from the cobalt-coordinated organic ligands [7] and, together with the size of the cobalamin core, underlies the need for multi-component systems to mediate controlled translocation and delivery of B12 across the cell membrane to its target enzyme [8].

Although bioinformatic analyses have predicted alternative vitamin transporters [9], BtuCD–BtuF remains the only confirmed bacterial B12 transport system identified to date [10]. The *Escherichia coli* model is the best characterized: a high-affinity corrinoid transporter, BtuB, operates with the TonB–ExbBD complex to traffic B12 across the outer membrane [11] where it is captured by the cytoplasmic membrane [12]. The reactivity of B12 cofactors derives from the cyan group in vitamin B12 (cyanocobalamin, CNCbl) must be replaced by deoxyadenosine and methyl ligands, respectively, during conversion to the biologically active cofactors: adenosylcobalamin (AdoCbl or coenzyme B12), which is required by methylmalonyl-CoA mutase, and methylcobalamin (MeCbl), which serves as an intermediary in the synthesis of methionine from homocysteine and methyltetrahydrofolate [6]. The reactivity of B12 cofactors derives from the cobalt-coordinated organic ligands [7] and, together with the size of the cobalamin core, underlies the need for multi-component systems to mediate controlled translocation and delivery of B12 across the cell membrane to its target enzyme [8].

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In this study, we used random mutagenesis to identify genes whose disruption abrogated the ability of *M. tuberculosis* to use exogenous vitamin B12 *in vitro*. Our results establish an essential role in B12 uptake for Rv1819c, a predicted ABC protein implicated in chronic infection *in vivo* [16], thereby revealing an alternative to the well-characterized BtuCD system for B12 transport.

### 3. Material and methods

#### 3.1. Bacterial strains and growth conditions

Strains, plasmids and oligonucleotides are described in the electronic supplementary material, table S1. *Mycobacterium tuberculosis* was grown on Middlebrook 7H10 (Difco) supplemented with 0.5 per cent glycerol and Middlebrook OADC enrichment (Difco) or in Middlebrook 7H9 supplemented with 0.2 per cent glycerol, Middlebrook OADC and 0.05 per cent Tween 80 or 0.05 per cent tyloxapol, as required. For propionate utilization experiments, 7H9 broth was supplemented with 0.5 per cent bovine serum albumin fraction V (Sigma), 0.085 per cent NaCl and 0.1 per cent (w/v) sodium propionate, as described [1]. Hygromycin (hyg), kanamycin (kan) and gentamicin (gent) were used at 50, 25 and 2.5 μgml⁻¹, respectively, CNCbl and AdoCbl at 10 μgml⁻¹, (CN)₂Cbl at 1 μM and 3-nitropropionate (3NP) at 0.1 mM.

#### 3.2. Construction of transposon mutant library

A library of transposon (Tn) mutants was constructed in *M. tuberculosis* H37Rv Δ*metH*, using the MycoMarT7 phage as described [17]. For the primary screen, transductants were plated across multiple 7H10 plates containing 20 μgml⁻¹ kan and 10 μgml⁻¹ CNCbl at a density of 20 000 colony forming units (CFU) per plate. The secondary screen was performed in duplicate in microtitre plate format and, for each Tn mutant, comprised four parallel wells containing 0.1 per cent propionate plus 20 μgml⁻¹ kan as base medium in each well: the first well constituted a growth control and contained only the base medium; in well 2, 10 μgml⁻¹ CNCbl was added to the base medium; in well 3, the base medium was supplemented with 0.1 mM 3NP; and in well 4, 0.1 mM 3NP and 10 μgml⁻¹ CNCbl were added.

#### 3.3. Identification of transposon insertion sites

A combination of Tn-linker [18] and rescue cloning [19] strategies was applied to identify Tn insertion sites using the oligonucleotides in the electronic supplementary material, table S1.

#### 3.4. Construction of mutant strains of *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* mutants were constructed using suicide plasmids described in electronic supplementary material, table S1. Genetic complementation used tweety-based vectors [20].
3.5. DNA sequencing

*Mycobacterium tuberculosis* genomic DNA was sequenced using an Illumina GenomeAnalyzer II, as described previously [21].

3.6. Homology modelling

The initial detection of crystal structures related to Rv1819c was performed using HHsearch [22] and COMA [23]. The Rv1819c model was then generated using a previously described iterative approach [24,25]. Briefly, both the set of structural templates and corresponding alignments were refined until the resulting model stopped improving and the visual inspection revealed no significant flaws.

4. Results

4.1. A forward genetic screen identifies $B_{12}$ uptake mutants

We showed previously that deletion of the $B_{12}$-dependent methionine synthase, MetH, renders *M. tuberculosis* sensitive to vitamin $B_{12}$ during growth on solid medium [2]. This phenotype depends on the function of a $B_{12}$ riboswitch that is located immediately upstream of *metE*, the gene encoding an alternative, $B_{12}$-independent methionine synthase in *M. tuberculosis*. In wild-type *M. tuberculosis*, exogenous $B_{12}$ suppresses transcription of *metE* by binding to the riboswitch [2], possibly ensuring efficient $B_{12}$-dependent methionine synthesis by MetH. In the *metH* deletion mutant, however, riboswitch-mediated suppression of *metE* in response to $B_{12}$ effectively results in the complete shutdown of methionine synthase activity, thereby eliminating production of an essential amino acid and so inhibiting bacillary growth [2]. This effect is most profoundly manifest on solid medium, where exposure to $10 \mu g \text{ml}^{-1}$ CNcbl results in a $3\log_{10}$-fold reduction in viable CFU of *ΔmetH* knockout mutants [2]. Here, we exploited the observed $B_{12}$ sensitivity of *metH* mutants in a genetic screen designed to elucidate a potential $B_{12}$ transport system in *M. tuberculosis* (figure 2). To this end, we constructed an unmarked *metH* knockout of the laboratory strain, *M. tuberculosis* H37Rv [21] (electronic supplementary material, figure S1a) and confirmed that it phenocopied the previously described hygromycin (*hgy*)-marked *ΔmetH* (BB) deletion mutant [2] during growth on $B_{12}$-containing solid medium (see the electronic supplementary material, figure S1b). The unmarked *ΔmetH* knockout was used as background strain in which to generate a Tn mutant library using the MycoMarT7 phage [17] that carries a kan resistance marker and inserts randomly at TA dinucleotides [19]. In the primary screen, the library of insertion mutants was plated on solid medium containing kan and CNcbl to enable the identification of genes whose disruption alleviated the growth defect of the *metH* mutant (figure 2a). In total, 612 individual clones were isolated, each of which was picked and regrown in standard liquid medium; of these, 35 grew poorly or not at all and were eliminated, leaving 577 'B$_{12}$-resistant' insertion mutants for further analysis.

Previously, in characterizing the *ΔmetH* (BB) mutant, we noted the high frequency at which suppressor mutants arose spontaneously on $B_{12}$-containing solid medium, with single-nucleotide polymorphisms (SNPs) in the *B$_{12}$* riboswitch located upstream of *metE* accounting for approximately 10–20 per cent of these [2]. In the current screen, we used dual selection on kan and CNcbl in order to limit the potentially confounding effects of spontaneous riboswitch mutations: according to these criteria, growth on CNcbl plus kan would require successful transduction with the kan-resistant Tn as well as disruption—

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**Figure 2.** Identification of genes required for $B_{12}$ transport and assimilation. (a) Schematic of the screening cascade. The $ΔmetH$ Tn library was plated on selective medium containing $10 \mu g \text{ml}^{-1}$ CNcbl. 612 $B_{12}$-resistant 'clones' were isolated and regrown in standard liquid medium, eliminating 35 mutants owing to poor ($n = 14$) or absent ($n = 21$) growth. A secondary screen tested the $B_{12}$ uptake ability of the remaining 577 insertion mutants in a four-well microtitre assay using 0.1% propionate (Prop) plus 20 $\mu g \text{ml}^{-1}$ kanamycin as base medium (well 1) supplemented with 10 $\mu g \text{ml}^{-1}$ CNcbl (well 2), 3NP (well 3) and 3NP plus 10 $\mu g \text{ml}^{-1}$ CNcbl (well 4). A total of 84 mutants failed to grow in well 4, suggesting impaired $B_{12}$ uptake. Each determination was performed in duplicate, and the results confirmed in batch culture. (b) Insertion mutants with disrupted $B_{12}$ uptake ability.

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**Table 1.** Tn insertion mutants from the primary screen.

| Tn insertion | total number |
|-------------|-------------|
| Rv1819c     | 72          |
| pduO        | 6           |
| mymA        | 2           |
| Rv2927c     | 1           |
| rpfB        | 1           |
| mutA        | 1           |
| unidentified| 1           |
4.2. Disruption of \( Rv1819c \) eliminates the ability of \( M. \) \( tuberculosis \) \( \Delta \) \( metH \) to use corrinoids for growth in 0.1% propionate-containing 3NP. Data are from a representative experiment performed in duplicate.

![Figure 3](image)

**Figure 3.** Disruption of \( Rv1819c \) eliminates the ability of \( M. \) \( tuberculosis \) \( \Delta \) \( metH \) to use corrinoids for growth in 0.1% propionate-containing 3NP. Data are from a representative experiment performed in duplicate.

...spontaneous or Tn-mediated—of \( B_{12} \)-dependent growth inhibition. Nevertheless, we predicted that a significant proportion of \( B_{12} \)-resistant mutants might contain Tn insertions in the riboswitch motif. So, in order to minimize the impact of disruptions to the \( B_{12} \) riboswitch, we applied a secondary screen (figure 2a) to determine the capacity of the insertion mutants to assimilate exogenous CNCbl for growth in liquid medium containing propionate in the presence of 3NP, an inhibitor of the key methylcitrate cycle enzyme, isocitrate lyase [26]. Two prior observations informed the design of this screen: (i) the inhibitory effect of genetic (prpDC) or chemical (3NP) abrogation of methylcitrate cycle enzymes during growth in liquid medium containing propionate can be alleviated by supplementing the culture with CNCbl, thereby enabling \( M. \) \( tuberculosis \) to use propionate as a carbon source via the methylmalonyl pathway that includes the \( B_{12} \)-dependent methylmalonyl-CoA mutase, MutAB [1]; (ii) for reasons that are not clear, \( B_{12} \)-mediated growth inhibition is less effective in liquid versus solid medium—that is, the \( \Delta \) \( metH \) mutant can grow in \( B_{12} \)-supplemented liquid medium (see the electronic supplementary material, figure S1c).

The secondary screen therefore assessed the ability of all 577 Tn mutants to use exogenous CNCbl for growth in liquid medium containing propionate in the presence of 3NP (figure 2a). The majority of Tn mutants (\( n = 493 \)) phenocopied the parental \( \Delta \) \( metH \) strain in this assay, and were eliminated as candidate \( B_{12} \) uptake mutants. In contrast, the remaining 84 Tn mutants were unable to grow in well 4, suggesting impaired ability to use exogenous \( B_{12} \) for methylmalonyl pathway-dependent propionate catabolism. To verify these results, 43 of the 84 mutants were selected at random for phenotypic confirmation of disrupted \( B_{12} \) uptake in batch culture (data not shown) and on \( B_{12} \)-containing solid medium (see the electronic supplementary material, figure S2a).

Insertions in \( Rv1819c \) accounted for 72 of the 84 Tn mutants (figure 2b; electronic supplementary material, figure S2a–c) and, moreover, mapped throughout the 1920 bp gene (electronic supplementary material, figure S2d). This result strongly suggested a role in \( B_{12} \) uptake for a predicted ABC transport protein previously identified as the putative \( M. \) \( tuberculosis \) homologue of BacA [16,27], a protein of cryptic function implicated in chronic infection in multiple host–pathogen models [25]. In their study of \( M. \) \( tuberculosis \) \( \Delta \) \( bacA \) in \( Rv1819c \), Domenech et al. [16] constructed a deletion mutant by allelic exchange mutagenesis (see the electronic supplementary material, figure S2b). We assessed the ability of this mutant—referred to as \( \Delta \) \( bacA::hyg \) by Domenech et al. [16]—to use exogenous \( B_{12} \) for MutAB-dependent growth in propionate (electronic supplementary material, figure S3a). Consistent with the inferred role of \( Rv1819c \) in \( B_{12} \) uptake, the \( \Delta \) \( bacA::hyg \) strain grew very poorly in propionate plus 3NP supplemented with \( B_{12} \), reproducing the phenotype of the \( \Delta \) \( metH \) \( Rv1819c::Tn \) mutants (figure 3). By contrast, the complemented derivative carrying a full-length copy of \( Rv1819c \) at the \( attB \) site, referred to as \( \Delta \) \( bacA::KLMH5 \) in the original study [16], was able to use \( B_{12} \) for growth (see the electronic supplementary material, figure S3a).

Similarly, integration of full-length \( Rv1819c \) at \( attB \) restored the \( B_{12} \)-sensitive phenotype of a randomly selected \( \Delta \) \( metH \) \( Rv1819c::Tn \) mutant during growth on solid medium supplemented with CNCbl (see the electronic supplementary material, figure S3b), and reversed the inability of the same mutant to use \( B_{12} \) for growth in propionate-containing liquid medium supplemented with 3NP (figure 3), confirming the essentiality of \( Rv1819c \) in this assay.

It was noticeable in the propionate utilization experiment (see the electronic supplementary material, figure S3a) that the \( \Delta \) \( bacA::hyg \) mutant started to replicate after two to three weeks of apparent growth arrest, possibly indicating the emergence of suppressor mutants. To circumvent this complication, we deleted the \( prpDC \) locus [28] in this strain, thereby negating the need to use 3NP to eliminate methylcitrate pathway function [1]. In contrast to the single \( prpDC \) deletion mutant, the double \( \Delta \) \( bacA::hyg \Delta \) \( prpDC \) knockout exhibited no growth at all in propionate over the 28-day time course (see the electronic supplementary material, figure S3c), even when supplemented with CNCbl, strongly suggesting that \( Rv1819c \) is required for the assimilation of exogenous \( B_{12} \) to enable methylmalonyl-CoA pathway function.
4.3. Spontaneous B12-resistant mutants carrying non-synonymous single-nucleotide polymorphisms in Rv1819c

We reported previously that SNPs in the metE-associated B12 riboswitch accounted for 10–20 per cent of all B12-resistant mutants isolated after plating the ΔmetH (BB) knockout on medium containing CNCbl, whereas the remaining B12-resistance mutations were unknown [2]. To investigate the possibility that mutations in Rv1819c might account for B12 resistance in those clones lacking riboswitch mutations, we plated the ΔmetH (BB) strain on medium containing CNCbl and sequenced the riboswitch region and Rv1819c locus in 10 spontaneous B12-resistant mutants. Consistent with previous results [2], two isolates carried independent mutations in the highly conserved B12-box motif within the metE riboswitch [29], namely C→T transitions at positions −155 and −163 relative to the metE start codon, respectively. Notably, four other B12-resistant mutants had wild-type riboswitch sequences, but contained non-synonymous SNPs in Rv1819c (see the electronic supplementary material, table S2), supporting the inferred role of Rv1819c in B12 uptake. To eliminate the possibility that an additional, unidentified mutation (or mutations) might account for the observed phenotype, we sequenced the genome of a representative Rv1819c point mutant, SP09 (see the electronic supplementary material, table S2). The parental, B12-sensitive strain, ΔmetH (BB), was differentiated from the laboratory strain, H37RvJO [21], only in the targeted deletion of metH sequence. Moreover, the Rv1819c mutation constituted the sole polymorphism separating SP09 from its ΔmetH (BB) parent and, importantly, complementation with wild-type Rv1819c at the attB locus restored B12 sensitivity to both SP09 and SP18 (see the electronic supplementary material, figure S4).

In the primary Tn screen (figure 2a), 'B12-resistant' mutants had been selected on kan and CNCbl in order to limit the potentially confounding effects of spontaneous riboswitch mutations. To verify the utility of this approach, we analysed the insertion sites in a random selection of 20 of the 493 ΔmetH Tn mutants subsequently eliminated in the secondary screen owing to their inability to use exogenous B12 for growth in propionate. All 20 mutants contained insertions in the B12 riboswitch region directly upstream of metE (data not shown), confirming that disrupted riboswitch function represents a major mechanism for loss of B12 regulation in strains which carry an intact Rv1819c gene.

4.4. Rv1819c is essential for corrinoid transport in Mycobacterium tuberculosis

Mycobacterium tuberculosis is predicted to encode a complete pathway for B12 biosynthesis, including enzymes required for the conversion of the B12 precursor, cobinamide, to AdoCbl through the addition of dimethylbenzimidazole and deoxyadenosine ligands [3]. The E. coli corrinoid transporter, BtuFCD, mediates uptake of cobinamide as well as CNCbl and AdoCbl [30], suggesting that Rv1819c might fulfil a corresponding role in M. tuberculosis. In support of this idea, cobinamide—provided as the dicyanide salt, (CN)2Cbi—was unable to complement the growth defect of ΔmetH Rv1819c::Tn mutants in propionate in the presence of 3NP, mimicking similar observations with AdoCbl and CNCbl (figure 3). Insertions in Rv1819c also alleviated the growth inhibitory effect of AdoCbl, CNCbl and (CN)2Cbi on the metH knockout mutant on solid medium, a phenotype that was reversed upon complementation with wild-type Rv1819c (see the electronic supplementary material, figure S5). In combination, these results confirmed the essentiality of Rv1819c for corrinoid transport in M. tuberculosis.

4.5. Impaired vitamin B12 uptake in spontaneous bleomycin-resistant Rv1819c mutants

Domenech et al. [16] showed that deletion of Rv1819c decreases the susceptibility of M. tuberculosis to the glycopeptide antibiotic, bleomycin, a phenotype commonly associated with BacA function [31–33]. We determined the minimum inhibitory concentration (MIC) of bleomycin against a selected Rv1819c::Tn mutant (electronic supplementary material, figure S6a) as well as the spontaneous B12-resistant mutants, SP09 and SP18 (see the electronic supplementary material, figure S6b), and observed values comparable to that reported for ΔbacA::jug [16]. To explore further the overlap between B12 uptake and bleomycin susceptibility, we isolated spontaneous bleomycin-resistant (Bleo<sup>a</sup>) mutants in two different genetic backgrounds, ΔprpDC and the unmarked metH knockout, by plating the strains on solid medium containing 3 μg ml<sup>−1</sup> bleomycin (10 × MIC). Five Bleo<sup>a</sup> mutants each of the ΔprpDC and ΔmetH knockouts were selected at random, and shown to be defective in their ability to use B12 for MutAB-dependent growth in propionate (see the electronic supplementary material, figure S7a). Moreover, the spontaneous Bleo<sup>a</sup> mutants of ΔmetH were resistant to CNCbl during growth on solid medium (see the electronic supplementary material, figure S7b). All five Bleo<sup>a</sup> mutants derived from the ΔprpDC strain carried nonsense mutations in Rv1819c, whereas missense mutations in Rv1819c were identified in four of five spontaneous Bleo<sup>a</sup> ΔmetH mutants (see the electronic supplementary material, table S2). Moreover, complementation with full-length Rv1819c reversed the inability of the spontaneous Rv1819c point mutants of ΔprpDC to catabolize propionate in liquid medium supplemented with CNCbl (figure 4), and restored the bleomycin susceptibility of SP09 to wild-type levels (see the electronic supplementary material, figure S6c).

4.6. Rv1819c encodes an ATP-binding cassette-type transporter

Rv1819c was previously included in a group of 'BacA-related' proteins identified on the basis of their similarity to the highly conserved BacA and SbmA proteins of Sinorhizobium and E. coli, respectively [27]. Unlike BacA/SbmA orthologues, however, which are predicted to require an interaction with a separate cytoplasmic protein for function, Rv1819c encodes both transmembrane (TMD) and nucleotide-binding (NBD) domains of an ABC transport protein on a single polypeptide. Sequence similarity analyses using only the TMD located M. tuberculosis Rv1819c in a cluster distinct from BacA/SbmA (see the electronic supplementary material, figure S8a). Moreover, these analyses indicated that Rv1819c was more closely related to ABC proteins other than BacA/SbmA in both E. coli and Sinorhizobium, namely YddA [34] and ExsE [35], respectively. The Rv1819c NBD similarly identified YddA
and ExsE as close homologues in an equivalent similarity search (see the electronic supplementary material, figure S8b), together with the recently described human ABC-type B\(_{12}\) transporter, ABCD4 [36].

We built a homology model of Rv1819c based on the crystal structures of two polyspecific ABC exporters, Staphylococcus aureus Sav1866 [37] and Salmonella typhimurium MsbA [38]. Consistent with known ABC protein architecture [39], Rv1819c is predicted to form a homodimer (figure 5), with each subunit comprising an N-terminal TMD fused to a highly conserved NBD that features all the motifs characteristic of functional ABC transporters (see the electronic supplementary material, figure S9a). Unlike Sav1866 and MsbA, though, the TMD domain of Rv1819c possesses an extra N-terminal region which is predicted to contain an additional transmembrane helix (see the electronic supplementary material, figure S9b). Proteomic analyses in the closely related M. bovis BCG suggest that this region is present in the mature protein [40], and therefore is not a signal peptide. However, in the absence of a close structural template containing seven transmembrane helices, we omitted the first 65 N-terminal residues in building the Rv1819c model. The predicted structure nevertheless provides a useful framework for the interpretation of experimental data. Notably, all three SNPs which resulted in substituted amino acids in the spontaneous B\(_{12}\)-resistant and Bleo\(^R\) mutants (see the electronic supplementary material, table S2) affect residues located in conserved regions of Rv1819c (figure 5). While the structural consequences of the P349T and G411D mutations require further investigation, L442S affects a conserved position in the putative nucleotide-binding pocket formed by two interacting ABC domains. In Sav1866, the corresponding residue, Ile356, makes a van der Waals contact with the sugar moiety of the bound ADP [37], and so supports the inferred association between a distorted pocket and crippled protein function.

4.7. A PduO-type adenosyltransferase is required for assimilation of vitamin B\(_{12}\)

CNCB has been shown to be required for the active cofactor, AdoCbl [41] (figure 6a). The genome of M. tuberculosis contains Tn insertions in pduO [42,43]. The genome of M. tuberculosis Rv1819c (PDB code: 3B60) [38], both of which contain transmembrane- (green) and nucleotide-binding (blue) domains fused into a single polypeptide chain that interacts to form a homodimer in the active protein. The two subunits in both structures are denoted by the different colour intensities. ADP molecules bound to each subunit are shown in purple and light purple, respectively. Residues substituted in spontaneous Rv1819c mutants are indicated with red arrows.

Figure 4. Impaired B\(_{12}\) uptake in a spontaneous bleomycin-resistant (Bleo\(^R\)) prpDC mutant, PBSP04, carrying a SNP in Rv1819c (see the electronic supplementary material, table S2). Strains were grown in 0.1% propionate supplemented with 3NP and CNCbl. Data are from a representative experiment performed in duplicate.

Figure 5. Rv1819c encodes an ABC-type transporter. Computational model of M. tuberculosis Rv1819c (amino acid residues 66–639) compared with the x-ray structure of Staphylococcus aureus Sav1866 (PDB code: 2HYD) [37]. The model is based on the crystal structures of Sav1866 and the ABC lipid flippase, MsbA, from Salmonella typhimurium (PDB code: 3B60) [38], both of which contain transmembrane- (green) and nucleotide-binding (blue) domains fused into a single polypeptide chain that interacts to form a homodimer in the active protein. The two subunits in both structures are denoted by the different colour intensities. ADP molecules bound to each subunit are shown in purple and light purple, respectively. Residues substituted in spontaneous Rv1819c mutants are indicated with red arrows.

5. Discussion

Our results identify Rv1819c as sole corrinoid transporter in M. tuberculosis under standard in vitro conditions and, moreover, establish the capacity of the organism to scavenge...
cinnamoyl-CoA reductase. This is a cytosolic enzyme that catalyzes the conversion of cinnamoyl-CoA to benzyldieneacetone (M. tuberculosis) or benzyldieneacetone (in Escherichia coli). The reaction involves the reduction of the double bond in the aromatic ring of the cinnamoyl group, which is catalyzed by a flavin monooxygenase enzyme.

The membrane-bound acyl-CoA dehydrogenase (ACAD) is another enzyme involved in the cinnamoyl-CoA metabolism. ACAD catalyzes the removal of the acetyl group from the cinnamoyl-CoA to produce cinnamoylcarnitine. This reaction is important in the disposal of cinnamoyl-CoA by the enzyme cinnamoylcarnitine translocase (CLT), which is responsible for transporting cinnamoylcarnitine across the mitochondrial membrane. The transport of cinnamoylcarnitine is facilitated by the mitochondrial carrier protein (MCT).

In summary, the metabolism of cinnamoyl-CoA involves several enzymes, including cinnamoyl-CoA reductase, acyl-CoA dehydrogenase, and cinnamoylcarnitine translocase, which work together to ensure the proper disposal of cinnamoyl-CoA and maintain cellular homeostasis.

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**Figure 6.** PduO is essential for B12 salvage and assimilation. (a) Predicted steps in late-stage AdoCbl biosynthesis and salvage in *M. tuberculosis*. Cobinamide is provided *in vitro* as a dicyanide salt, (CN)$_2$Cbi. (b) The ΔmetH pduO::Tn mutant cannot use CNCbl or (CN)$_2$Cbi for growth in 0.1% propionate-containing 3NP. Data are from a representative experiment performed in duplicate.
structure suggests that compromised B12 uptake in these strains excluded spontaneous mutations as the underlying cause of the observed B12 phenotypes. A single mutant carried an insertion in rpfB, which encodes a resuscitation-promoting factor. To explore this result further, we retested ΔmetH rpfB::Tn in parallel with an rpfB deletion mutant of H37Rv, constructed previously [62]. Although the Tn mutant was not able to use exogenous CNcbl for growth in propionate-containing medium, the ∆rpfB knockout strain phenocopied wild-type H37Rv in this assay (data not shown), thereby excluding a role for RpfB in B12 uptake. It is possible that rpfB::Tn possesses an additional, unidentified polymorphism, affecting B12 assimilation; alternatively, polar effects on the downstream gene, ksgA, encoding dimethyladenosine transferase, might contribute to the observed phenotype [63], a possibility under investigation. Two additional Tn insertions mapped to mymA, encoding a putative flavin-dependent monoxygenase. The predicted role of MymA in the maintenance of cell wall ultrastructure [64] suggests that compromised B12 uptake in these mutants might be non-specific; however, this requires further investigation, and is complicated by the fact that mymA is the first gene in a seven-gene operon [65]. We also isolated a mutA::Tn mutant, whose inability to use propionate for growth in B12-containing medium is consistent with impaired methylmalonyl-CoA mutase function. The basis for the B12 resistance of this mutant in the primary screen is unclear, however, and probably also the result of an additional spontaneous mutation. The final Tn insertion mapped to Rs2927c, a gene which previous saturation mutagenesis studies have predicted as essential for growth of M. tuberculosis in vitro [66,67]. Although the function of Rs2927c has been proposed to operate as part of the cell division machinery [68], it seems probable that, like the mymA::Tn mutants, the failure of Rs2927c::Tn to assimilate B12 is non-specific. However, given the inferred requirement for PduO-dependent adenosylation in the assimilation of exogenous B12, the prediction that Rs2927c might function in de novo adenosine nucleotide biosynthesis [69] is intriguing, and the subject of current investigation.

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