A Riboswitch Regulates Expression of the Coenzyme B₁₂-Independent Methionine Synthase in *Mycobacterium tuberculosis*: Implications for Differential Methionine Synthase Function in Strains H37Rv and CDC1551[†]

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We observed vitamin B₁₂-mediated growth inhibition of *Mycobacterium tuberculosis* strain CDC1551. The B₁₂ sensitivity was mapped to a polymorphism in *metH*, encoding a coenzyme B₁₂-dependent methionine synthase. Vitamin B₁₂-resistant suppressor mutants of CDC1551 containing mutations in a B₁₂ riboswitch upstream of the *metE* gene, which encodes a B₁₂-independent methionine synthase, were isolated. Expression analysis confirmed that the B₁₂ riboswitch is a transcriptional regulator of *metE* in *M. tuberculosis*.

Comparative genomics has been profitably utilized to elucidate interstrain variability between *Mycobacterium tuberculosis* clinical and laboratory isolates (7, 8, 19, 20). Moreover, recent studies (11, 14, 16) have begun to address the next challenge: mapping phenotypic consequences to identified polymorphisms. A whole-genome comparison between H37Rv, a virulent laboratory strain, and the clinical isolate CDC1551 identified a large sequence polymorphism at the *PPE37-metH* locus in CDC1551 that eliminates 1,196 bp at the 3' terminus of the *metH* gene (7) (Fig. 1). MetH is one of two methionine synthases predicted to catalyze the final S-methyl transfer reaction in de novo methionine biosynthesis in *M. tuberculosis* (4)—the formation of methionine from homocysteine—and requires a vitamin B₁₂-derived cofactor for activity; the other, MetE, does not. Full-length MetH is a monomer comprising four distinct domains (Fig. 1). In CDC1551, 398 amino acids have been lost from the C terminus, partially disrupting the coenzyme B₁₂ (or cobalamin [CBL])—binding domain and eliminating completely the S-adenosyl-L-methionine (SAM)—binding domain, which is required for the reductive reactivation of the B₁₂ moiety in MetH (6).

Little is known about the regulation of corresponding B₁₂-dependent and B₁₂-independent enzymes in *M. tuberculosis* or their contribution to viability and pathogenesis (5). However, any suggestion of redundant methionine synthase function must be reconciled with the predicted essentiality of *metE* for optimal growth of *M. tuberculosis* in vitro (18). In particular, the apparent inability of *metH* to compensate for the loss of *metE* raises fundamental questions about the functionality of

metH in this organism. Alternatively, the predicted essentiality of *metE* may indicate an inability of *M. tuberculosis* to produce the B₁₂ cofactor necessary to enable MetH to function as the sole methionine synthase in vitro, despite encoding an apparently complete vitamin B₁₂ biosynthetic pathway (17).

In genomes containing corresponding B₁₂-dependent and B₁₂-independent enzymes, the activity of the B₁₂-independent enzyme is often subject to regulation by a B₁₂ riboswitch (23). Riboswitches are highly structured domains found within the mRNA of the regulated gene (1). Ligand-specific binding of a small molecule (such as B₁₂) to the riboswitch results in the formation of an alternative RNA structure that attenuates transcription or translation (12). The association of riboswitch-mediated regulation with many essential metabolite biosynthetic and transport pathways has prompted the development of novel antibacterials that target these genetic control elements (2). Two B₁₂ riboswitch motifs have been identified in the *M. tuberculosis* genome and are located immediately upstream of *metE* (Fig. 2) and *PPE2* (17, 23). Recently, Borovok et al. (3) took advantage of a B₁₂ riboswitch to effect growth

FIG. 1. Deletion polymorphism at the *PPE37-metH* locus of CDC1551. Full-length MetH comprises an N-terminal homocysteine-binding domain (HCY), an N-methyltetrahydrofolate-binding domain (MTH), a CBL-binding domain, and a C-terminal SAM-binding domain (6). A large sequence polymorphism eliminates the SAM-binding domain in CDC1551 MetH (shaded box). Genomic regions deleted in the respective H37Rv *metH* mutants are also shown (open boxes).

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FIG. 2. Predicted *M. tuberculosis metE* B₁₂ riboswitch. The secondary structure is drawn according to the scheme presented by Vitreschak et al. (23). Uppercase letters indicate residues identified by Vitreschak et al. (23) to be invariant across approximately 200 B₁₂ riboswitches from 67 bacterial genomes; lowercase letters are *M. tuberculosis* specific. The conserved B₁₂-box is highlighted. The square and circle denote sites of C→T transition mutations identified in B₁₂ suppressor mutants of CDC1551 and H37Rv Δ *metH*(B), respectively. The triangles denote single-nucleotide polymorphisms identified in the B₁₂ riboswitch upstream of the *nrdABS* operon in B₁₂ suppressor mutants of *S. coelicolor* (3) (see the text for details).

arrest in a *Streptomyces coelicolor* mutant lacking the coenzyme B₁₂-dependent ribonucleotide reductase (RNR) NrdJ. In that case, transcription of the corresponding B₁₂-independent enzyme, NrdAB, was repressed by exogenous B₁₂, effectively abrogating essential RNR function in *Streptomyces*.

We sought to address by genetic means whether *metH* is functional in *M. tuberculosis* H37Rv and to investigate the consequences of the deletion polymorphism on the function of *metH* in CDC1551. Furthermore, the location of a putative riboswitch in the *metE* promoter region of *M. tuberculosis* suggested the possibility of exploiting the CDC1551 *metH* polymorphism to determine whether B₁₂ regulates the activity of *metE* in *M. tuberculosis*. In this paper, we describe the results of these investigations.

A *metE* mutant of *M. tuberculosis* H37Rv is viable when supplemented with vitamin B₁₂. To establish the ability of *metH* to compensate for the loss of *metE* in *M. tuberculosis*, we attempted to generate a *metE* deletion mutant of H37Rv (Δ *metE*) (Fig. 3A) by allelic replacement with a *hyg*-marked *metE* deletion allele carried on the suicide plasmid p2 Δ *metE*17 (see Table S1 in the supplemental material) by previously described methods (9, 15). Consistent with the predicted essentiality of *metE* (18), a Δ *metE* mutant could not be obtained on normal solid medium (Middlebrook 7H10; Difco). However, supplementing the growth medium with vitamin B₁₂ (cyanocobalamin; Sigma) at a concentration of 10 μ g/ml enabled the ready isolation of a Δ *metE* mutant (Fig. 3B and C). The successful generation of an H37Rv *metE* mutant on B₁₂-supplemented medium was significant, as it established simultaneously the ability of *M. tuberculosis* to transport vitamin B₁₂ despite the absence of identifiable B₁₂-specific transporters in the *M. tuberculosis* genome (17) and the capacity of the organ-

ism to convert exogenous vitamin B₁₂ into the methylcobalamin cofactor required for MetH-catalyzed S-methyl transfer (10). It also strongly supported functionality of the *metH*-encoded gene, although the need to supplement the growth medium suggested that *M. tuberculosis* was unable to produce sufficient B₁₂ cofactor in vitro to allow MetH to compensate for the loss of function of MetE.

Growth of CDC1551 is inhibited on solid medium containing vitamin B₁₂. We reasoned that if the truncation in CDC1551 *metH* rendered this gene nonfunctional, then it should not be possible to construct a CDC1551 *metE* deletion mutant by the approach described above for H37Rv. Indeed, we were unable to recover a *metE* mutant of this strain using the knockout vector p2 Δ *metE*17 (see Table S1 in the supplemental material): 102 white, sucrose-resistant, hygromycin-resistant clones recovered from the two-step allelic exchange procedure (15) were screened by PCR, and all were found to retain the wild-type *metE* allele. The inability to disrupt *metE* in CDC1551 thus provided a further indication that its truncated *metH* gene was not functionally active.

During the course of these experiments, we noticed that wild-type CDC1551 exhibited a marked plating defect on solid medium supplemented with vitamin B₁₂ (Fig. 3C): at a B₁₂ concentration of 10 μ g/ml, a 3 log reduction in CFU was evident (Fig. 3B). In stark contrast, no effect on H37Rv growth was observed (Fig. 3B and C). These observations suggested that differential methionine synthase function might determine the contrasting growth phenotypes displayed by CDC1551 and H37Rv on Middlebrook 7H10 agar supplemented with B₁₂. That is, an inactivated *metH* coupled with B₁₂-mediated suppression of *metE* effectively abrogated all methionine synthase activity in CDC1551, thus preventing growth.

FIG. 3. Construction and phenotypic characterization of *M. tuberculosis* methionine synthase mutants. (A) Construction and genotypic characterization of mutant strains. Genomic DNA was digested with the relevant restriction enzyme and probed with either a *metE*- or *metH*-specific probe. Restriction maps of the various strains are illustrated schematically in the line drawings adjacent to each Southern blot (not drawn to scale). For *metE*, Southern blots were probed with a 1,989-bp fragment (*metE*_p, represented by a spotted box) containing 564 bp of 5'-terminal *metE* coding sequence obtained by PCR amplification using the primer pair *metEF2/metER2* (see Table S2 in the supplemental material). The $\Delta metE::hyg$ mutation eliminates 1,367 bp of *metE* coding sequence; however, an additional BamHI site is introduced by the *hyg* cassette (B, BamHI; WT, wild-type H37Rv). For *metH*, Southern blots were probed with the 644-bp MluI-BglII fragment of H37Rv *metH* (*metH*_p, represented by a hatched box). The probe corresponds to the SAM-binding domain of H37Rv *metH* and so fails to hybridize to CDC1551 genomic DNA. The $\Delta metH(B)$ mutation eliminates 391 bp of *metH* coding sequence, whereas the $\Delta metH(BB)$ mutation eliminates 1,417 bp *metH* coding sequence, including an MluI site (M, MluI; WT, wild-type H37Rv). *metH*Kin is a single-crossover homologous recombination mutant of CDC1551 containing both truncated CDC1551 and full-length H37Rv *metH* alleles. (B) Effect of exogenous vitamin B_{12} on growth of CDC1551 and H37Rv methionine synthase mutants. Cells were incubated on solid 7H10 medium containing 10 $\mu\text{g/ml}$ vitamin B_{12} (black bars), and CFU were scored after 4 weeks. Gray bars indicate normal medium without supplement. Data represent mean CFU from two independent experiments performed in duplicate, and error bars indicate standard deviations. The H37Rv $\Delta metE$ mutant requires B_{12} supplementation for growth on 7H10, ensuring an absence of CFU on unsupplemented medium; B12P2 is a representative CDC1551 B_{12} suppressor strain containing a mutated B_{12} riboswitch (see the text and Fig. 2). (C) Representative plates.

Disruption of *metH* renders H37Rv sensitive to vitamin B_{12} .

To confirm that the lack of MetH function was directly responsible for the B_{12} sensitivity of CDC1551, we constructed two independent *hyg*-marked *metH* deletion mutants of H37Rv (Fig. 3A; also see Table S1 in the supplemental material). A

391-bp BglII deletion in H37Rv $\Delta metH(B)$ eliminated 63 amino acids in the CBL domain and 71 amino acids in the SAM domain (Fig. 1), roughly approximating the CDC1551 *metH* genotype in which 102 amino acids are missing from the CBL domain together with the entire SAM domain. H37Rv

$\Delta metH(BB)$, on the other hand, contained a much larger deletion (1,417-bp region spanned by BglII and BclI sites), which eliminated the entire CBL domain and disrupted both the methyltetrahydrofolate and SAM domains (Fig. 1). The $\Delta metH(BB)$ allele was therefore expected to abrogate *metH* activity completely. Plating both mutants on medium supplemented with vitamin B₁₂ at 10 μ g/ml confirmed that disruption of *metH* indeed renders *M. tuberculosis* B₁₂ sensitive (Fig. 3B and C). Furthermore, $\Delta metH(B)$ recapitulated precisely the CDC1551 phenotype, indicating that the combined disruption of the CBL and SAM domains is sufficient to render *M. tuberculosis* MetH nonfunctional. Conversely, by introducing a copy of the full-length H37Rv *metH* gene into the CDC1551 chromosome, we were able to reverse the B₁₂ sensitivity of CDC1551 (*metH*Kin) (Fig. 3B and 3C). CDC1551 *metH*Kin (Fig. 3A) is a single-crossover recombinant that carries the full-length H37Rv *metH* open reading frame plus flanking and vector sequences (p2*metH*Kin) (see Table S1 in the supplemental material), as well as retaining its native truncated *metH* allele. Together, these observations provided further evidence that CDC1551 MetH was inactive.

Vitamin B₁₂-resistant CDC1551 mutants containing mutated B₁₂ riboswitches. Colonies of CDC1551 of normal size and morphology arose on B₁₂-containing plates at a frequency of $\sim 10^{-3}$ (Fig. 3B). We postulated that a mutation in the B₁₂ riboswitch motif upstream of *metE* might relieve B₁₂-mediated suppression of *metE* and so allow growth of these “vitamin B₁₂ suppressor” mutants. To ascertain the heritability of the suppressor phenotype, selected colonies were passaged in liquid medium (Middlebrook 7H9; Difco) without B₁₂ supplement. Restreaking of these cultures onto solid medium supplemented with B₁₂ established that the ability to grow on B₁₂ was not lost after passage (a representative suppressor mutant, B12P2, is shown in Fig. 3B and C). To characterize these mutants genotypically, we sequenced the 500-bp genomic region upstream of *metE*, which contains the B₁₂ riboswitch in 10 CDC1551 B₁₂ suppressor mutants. We also sequenced 10 B₁₂ suppressor mutants derived from H37Rv $\Delta metH(B)$. This analysis revealed that 2 of the 10 CDC1551 mutants contained a C→T transition in a conserved region of the B₁₂ riboswitch termed the “B12-box” (23). In addition, one H37Rv $\Delta metH(B)$ suppressor mutant contained a C→T transition in an adjacent position within the B12-box (Fig. 2). These data supported the idea that the vitamin B₁₂ plating deficiency of CDC1551 results from B₁₂-mediated repression of *metE*. However, the mechanism(s) of resistance in the remaining CDC1551 and $\Delta metH(B)$ B₁₂ suppressor isolates remains to be elucidated. Preliminary sequence analysis has failed to reveal any mutations in the only other identified *M. tuberculosis* B₁₂ riboswitch—located upstream of *PPE2* (17)—in these strains. Instead, it is possible that they represent B₁₂ transport mutants with diminished capacity to take up B₁₂.

The B₁₂ riboswitch is a transcriptional regulator of *metE*. To determine whether the B₁₂ riboswitch functions as a transcriptional or posttranscriptional regulator of *M. tuberculosis metE*, we analyzed the expression of *metE* during growth in liquid medium supplemented with vitamin B₁₂. Expression of the housekeeping gene *sigA* was used to benchmark relative expression levels, as described previously (5, 13). In both H37Rv and CDC1551, *metE* transcript levels were significantly re-

FIG. 4. Exogenous vitamin B₁₂ represses transcription of *M. tuberculosis metE*. Reverse transcription (RT)-PCR analysis of *metE* expression in strains H37Rv, CDC1551, and the B₁₂ suppressor mutant B12P2 is shown. Cells were grown for 5 h in Sauton's minimal medium in the presence (+) or absence (−) of 10 μ g/ml vitamin B₁₂ before being harvested for mRNA isolation. RT-PCR methods are described in the supplemental material, and primer sequences are detailed in Table S2. The data shown are representative of two independent experiments.

duced in B₁₂-supplemented medium, whereas expression of *sigA* was unaffected (Fig. 4). In contrast to the repression of *metE* observed in the parental CDC1551 strain, the expression of *metE* in the B12P2 mutant was completely unaffected by exogenous B₁₂. This observation confirmed that vitamin B₁₂-mediated repression of *metE* activity in wild-type *M. tuberculosis* occurs at the level of transcription. Interestingly, constitutive transcription of *metE* in the B12P2 mutant was noticeably diminished relative to H37Rv and CDC1551 when standardized against *sigA*. The B12P2 mutant displayed no growth deficiency under standard conditions in vitro, however, suggesting either that reduced *metE* expression has no effect on growth or that B12P2 harbors a second-site mutation(s) that ameliorates any growth-inhibitory effect of reduced *metE* expression.

Conclusions and implications. In this study, we took advantage of an identified deletion polymorphism in a clinical isolate to confirm fundamental aspects of *M. tuberculosis* biology. Specifically, we have presented genetic evidence that both *metE* and *metH* encode functional methionine synthases and that transcription of *metE* is controlled by a B₁₂ riboswitch. In addition, through the construction of a *metE* deletion mutant of strain H37Rv, we have demonstrated the ability of *M. tuberculosis* to transport vitamin B₁₂. A previous comparative genomic analysis had situated mycobacteria (including *M. tuberculosis*) in a rare category of B₁₂-utilizing organisms lacking an identifiable B₁₂-specific transport system (17). Further analysis of those B₁₂ suppressor isolates of CDC1551 and $\Delta metH(B)$ that did not map to the *metE*-related B₁₂ riboswitch may yield some insight into potential nonorthologous B₁₂ transport systems in *M. tuberculosis*.

The *M. tuberculosis* genome contains two other B₁₂-dependent enzymes in addition to *metH* (4): a class II RNR encoded by *nrdZ* (5) and a methylmalonyl coenzyme A mutase encoded by *mutAB*. The proof of functionality of both a B₁₂-dependent enzyme (MetH) and a B₁₂-dependent regulatory mechanism (the B₁₂ riboswitch), therefore, has broader implications for *M. tuberculosis* pathogenesis. For example, does *M. tuberculosis* synthesize B₁₂ and, if so, under what conditions? Alternatively, does *M. tuberculosis* acquire vitamin B₁₂ from the host? In either case, whether host acquired or synthesized de novo, does B₁₂ availability signal a shift in metabolism to the utilization of all three B₁₂-dependent enzymes? Also, given the presence of only two B₁₂ riboswitches in the *M. tuberculosis* genome, how is flux through the remaining B₁₂-dependent

pathways (NrdZ and MetAB) regulated? Moreover, why has CDC1551 retained an intact B₁₂ riboswitch motif upstream of *metE* despite the loss of *metH* function? This question, in particular, might inform the observation that only a small proportion (3 of 20) of sequenced vitamin B₁₂-resistant isolates of CDC1551 and H37Rv Δ *metH*(B) possessed mutated B₁₂ riboswitch motifs. Through the selective application of the methionine synthase mutants described here, as well as others containing disruptions in B₁₂ biosynthetic pathways, we are actively addressing these issues.

Finally, the disruption of *metH* in a clinical strain suggests the dispensability of this enzyme for *M. tuberculosis* pathogenesis. However, the effect that loss of the alternative methionine synthase has on the pathogenesis of *M. tuberculosis* remains to be determined. The epidemiology of disease caused by strain CDC1551 may be instructive: the strain was originally isolated as the highly infectious agent of a number of tuberculosis outbreaks (21, 22). Is it significant, therefore, that deletion polymorphisms affecting two of the three B₁₂-dependent enzymes (NrdZ and MetH) have been identified in individual *M. tuberculosis* clinical isolates (7, 19) that, by definition, are in active circulation? That is, does loss of B₁₂-dependent enzymes favor transmission and disease? In this regard, it is intriguing to note that preliminary sequence data from two other clinical isolates, strain C (*Mycobacterium tuberculosis* Sequencing Project, Broad Institute of Harvard and the Massachusetts Institute of Technology [http://www.broad.mit.edu]) and strain 210 (http://www.tigr.org), have revealed separate sequence polymorphisms in *metH*. The consequences of these polymorphisms for *metH* functionality are unknown; however, given that adaptive evolution of *M. tuberculosis* is dependent entirely on chromosomal rearrangements and mutations, at the very least these observations suggest that the *metH* locus may be under active selective pressure.

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