A tRNA modification in *Mycobacterium tuberculosis* facilitates optimal intracellular growth

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Abstract

Diverse chemical modifications fine-tune the function and metabolism of tRNA. Although tRNA modification is universal in all kingdoms of life, profiles of modifications, their functions, and physiological roles have not been elucidated in most organisms including the human pathogen, *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis. To identify physiologically important modifications, we surveyed the tRNA of *Mtb*, using tRNA sequencing (tRNA-seq) and genome-mining. Homology searches identified 23 candidate tRNA modifying enzymes that are predicted to create 16 tRNA modifications across all tRNA species. Reverse transcription-derived error signatures in tRNA-seq predicted the sites and presence of 9 modifications. Several chemical treatments prior to tRNA-seq expanded the number of predictable modifications. Deletion of *Mtb* genes encoding two modifying enzymes, TruB and MnmA, eliminated their respective tRNA modifications, validating the presence of modified sites in tRNA species. Furthermore, the absence of *mnmA* attenuated *Mtb* growth in macrophages, suggesting that MnmA-dependent tRNA uridine sulfation contributes to *Mtb* intracellular growth. Our results lay the foundation for unveiling the roles of tRNA modifications in *Mtb* pathogenesis and developing new therapeutics against tuberculosis.

Introduction

tRNA is an adaptor molecule that enables protein synthesis by converting the triplet genetic code in mRNA into amino acids. The fidelity of base paring between mRNA codons and tRNA anticodons is monitored within ribosomes and is critical for properly incorporating the amino acids bound to the 3' ends of tRNAs into growing polypeptides. For optimal translation, the abundances, and properties of tRNA isoacceptors are fine-tuned by diverse mechanisms, including chemical modifications[1-4] Dysregulation of tRNA abundance and/or structure leads to defective decoding and results in ribosome pausing and collisions, protein misfolding, stress responses and can have detrimental or lethal effects on the cell[5-10].

Chemical modifications of tRNA (tRNA modifications) are found in all kingdoms of life and fine-tune tRNA properties including mRNA decoding efficiency, recognition by aminoacyl-tRNA synthetases, half-life and structural stability[2, 10-12]. Modifications are prevalent in the anticodon loop, particularly at the first letter of the anticodon. Modifications of the anticodon loop directly modulate codon recognition, whereas modifications in the tRNA body region primarily stabilize tRNA tertiary structure, protecting them from degradation in the cell. tRNA modifications are generated by dedicated site-specific enzymes referred to as tRNA modifying enzymes. tRNA modifications have been extensively characterized in a few model organisms[2, 13], but their profiles, regulation, and functions in non-model organisms, including bacterial pathogens, are understudied[13]

tRNA sequencing (tRNA-seq) allows for the rapid and systematic prediction of many tRNA modification sites[14, 15]. We recently developed a comparative tRNA-seq protocol to profile tRNA modifications in organisms with uncharted tRNA modification profiles; in *Vibrio cholerae,* this approach led to the discovery of a new RNA modification and RNA editing process[16]. tRNA-seq enables rapid prediction of modified sites through detection of reverse transcription-derived signatures, such as nucleotide misincorporation and early termination, both of which occur more

52 frequently at modified sites. Furthermore, several chemical treatments of tRNA can convert
53 modifications that are not recognizable as reverse transcription-derived signatures into detectable
54 signals, expanding the repertoire of modifications that can be distinguished by tRNA-seq[17-20].

55 *Mycobacterium tuberculosis* (*Mtb*), the agent of tuberculosis (TB), is a global pathogen that caused
56 >10.5 million cases and over 1.5 million deaths worldwide in 2020[21]. Several studies have uncovered
57 roles for non-*Mtb* mycobacterial tRNA modifications in stress responses, adaptation to environmental
58 changes, and persister cell formation[22]. *Mycobacterium bovis* BCG, an organism closely related to *Mtb*,
59 responds to hypoxia by reprogramming 40 ribonucleoside modifications in tRNA to facilitate
60 translation of a subset of proteins that promote survival in hypoxic conditions[22]. To date, studies of
61 the profiles and functions of *Mtb* tRNA modifications have been limited.

62 Here, we conducted tRNA-seq in *Mtb*. We assigned modifications to many of the reverse
63 transcription-derived signatures identified, using information on the presence of homologs of known
64 modifying enzymes in *Mtb*. Chemical treatments of tRNAs carried out prior to tRNA-seq increased
65 the detectability of certain modifications. We constructed two *Mtb* deletion mutant strains, with
66 deletions of *mnmA* and *truB*, and confirmed that the absence of these modification enzymes eliminated
67 the predicted signals in tRNA-seq data. Furthermore, while deletion of *mnmA* in *Mtb* did not affect
68 the pathogen's growth in *in vitro* laboratory growth conditions, the *mnmA* knockout strain's growth
69 was attenuated in a macrophage infection model. Our findings suggest that tRNA modifications
70 warrant further study as we unravel the complexity of *Mtb* infections, as they may serve as targets for
71 new therapeutics.

72 **Results**

73 ***In silico* prediction of *Mtb* tRNA modifying enzymes**

74 To predict tRNA modifications in *Mtb*, we used Basic Local Alignment Search Tool
75 (BLAST)[23] to identify homologues of all tRNA modification enzymes registered in Modomics in
76 the *Mtb* genome[24] (Supplementary Table 1). With a stringent threshold (E-value $<1\times10^{-10}$), 31 *Mtb*
77 genes homologous to genes encoding known RNA modification enzymes were identified
78 (Supplementary Table 2). 23 of these genes are predicted to synthesize 16 tRNA modifications in *Mtb*
79 (Supplementary Table 2 and 3), including *miaA* and *miaB* for 2-methylthio-6-isopentenyl-adenosine
80 (ms^2i^6A), *tsaD*, *tsaB*, *tsaE*, and *sua5* for N^6-threonylcarbamoyladenosine (t^6A), *mnmA* for 2-thiouridine
81 (s^2U), *truB*, *truA*, *rluA*, and *pus9* for pseudouridine (Ψ), *trm2* for 5-methyluridine (m^5U), *trmD* for 1-
82 methylguanosine (m^1G), *trmI* for 1-methyladenosine (m^1A), *trmL* for 2'-*O*-methylcytidine (Cm) or 2'-
83 *O*-methyluridine (Um), *trmB* for 7-methylguanosine (m^7G), two *trmH* for 2'-*O*-methylguanosine (Gm),
84 *trm*R for 5-methoxyuridine (mo^5U), *trcM* for 5-methylcytidine (m^5C), *dusB* for dihydrouridine (D), *tadA*
85 for inosine (I), and *tilS* for lysidine (k^2C). While eight additional genes met the threshold for homology
86 to modification enzymes, they exhibited greater similarity to a Glutamyl-tRNA synthase; Rv2992c,
87 ribosome associated GTPases (Der; Rv1713 and Era; Rv2364c), molybdopterin biosynthesis proteins
88 (MoeW; Rv2338c and MoeB2; Rv3116), thiosulfate sulfur transferases (CysA; Rv3117 and SseA;
89 Rv3283) and a riboflavin biosynthesis protein (RibG; Rv1409), and likely do not correspond to tRNA
90 modification enzymes (Supplementary Table 2).

91 We mined data from genome-wide Tn-seq and CRISPRi screens[25, 26] to assess the impacts
92 of *Mtb* tRNA modifications on its growth. Five genes encoding *Mtb* tRNA modifying enzymes, *trmD*,
93 *tilS*, *tadA*, *tsaC2,* and *miaA*, were reported to be essential for *Mtb* growth in both Tn-seq[25] and
94 CRISPRi screens[26] (Supplementary Table 3), suggesting that the modifications they produce are
95 critical for tRNA functions. Indeed, *E. coli trmD, tilS, tadA, and tsaC2* are also essential for growth and

96 critical for codon decoding, aminoacylation, and reading frame maintenance[27-30]. The *Mtb*
97 modifications synthesized by these enzymes likely have similar impacts on tRNA functions.
98 Unexpectedly, one modifying enzyme, MiaA, is non-essential in *E. coli* grown in nutrient rich medium,
99 but apparently essential in *Mtb*, suggesting that i^6A, the modification introduced by MiaA, may have
100 more profound roles in *Mtb* translation than in *E. coli*.

101 Several of the putative *Mtb* tRNA modifying enzymes are conserved across all three domains
102 of life (e.g., TruB and TsaD) (Fig. 1). By contrast, some enzymes were limited to bacterial species
103 closely related to *Mtb*, possibly suggesting their species-specific physiological roles, including
104 pathogenesis. For example, TrmI (Rv2118c) homologs are widely present in Archaea and Eukaryotes,
105 but are sparsely distributed in bacteria, where they are primarily limited to Actinomycetia, including
106 *Mtb*, and several thermophilic bacterial species (e.g., *Thermus thermophilus*). TrmI synthesizes m^1A at
107 position 58 in eukaryotes[31] and at position 57/58 in archaea[32]; indeed, TrmI in *Mtb* has been
108 proven to generate m^1A at position 58[33]. Pus9 (Rv3300c) has many homologs exhibiting weak
109 similarity across eukaryotes and bacteria. However, some species, including *Pseudomonas aeruginosa*,
110 *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, and several species of Actinomycetia, encode proteins
111 showing strong homology to *Mtb* Pus9, suggesting that this set of pseudouridylases have a distinctive
112 property such as substrate specificity.

113 tRNA modifying enzymes predicted in *Mtb* were observed in mycobacteria species, including
114 *Mycolicibacterium smegmatis* and *Mycobacteroides abscessus* (Fig. 1), suggesting that tRNA modification
115 patterns are similar among mycobacterium species.

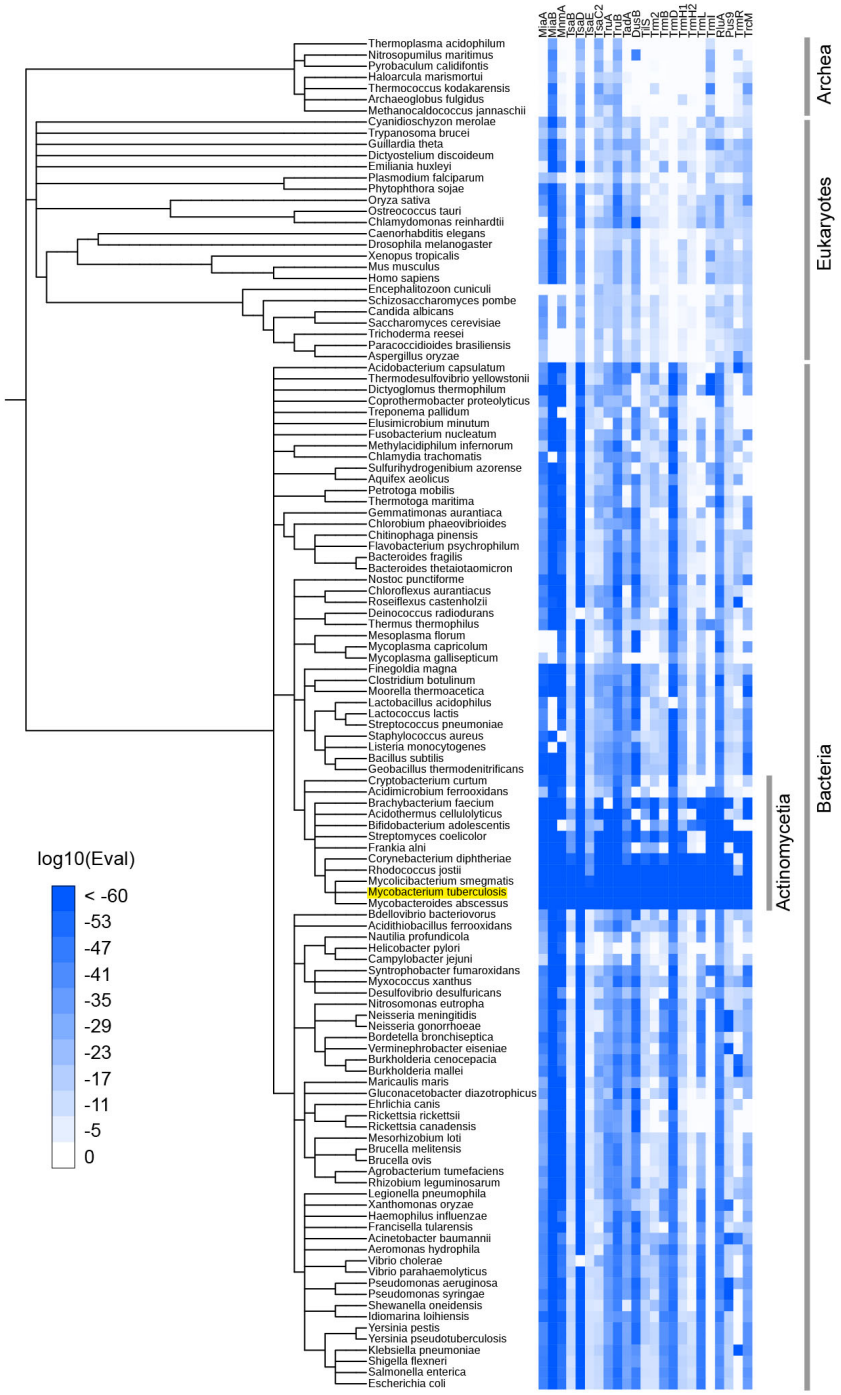

Fig. 1. Phylogenetic distribution of *Mtb* tRNA modifying enzyme homologs. Heat map of log10 E-values from BLAST search results. BLAST searches were conducted against 120 manually picked organisms using *Mtb* tRNA modifying enzymes as queries. When one organism has multiple hits, the lowest log10(Eval) values among hits is shown. iTol[34] was used to depict the results.

123 **Profiling *Mtb* tRNA modification sites by tRNA sequencing**

124 To begin profiling detectable *Mtb* tRNA modifications, we sequenced tRNAs isolated from
125 wild type *Mtb* strain H37Rv grown in 7H9 medium. In this protocol, tRNAs are first reversed
126 transcribed to cDNA[16]. During cDNA synthesis, chemical modifications on tRNA nucleotides
127 disrupt Watson-Crick base pairing and increase the frequency of reverse transcriptase errors, leading
128 to incorporation of the incorrect nucleotide or early termination of cDNA synthesis[35]. These
129 reverse transcription derived 'signatures' typically correspond to modified sites[14, 15] and are
130 depicted in the heat map in Fig. 2.

131 Comparison of the reverse transcription derived signatures observed in *Mtb* to *E. coli*, where
132 tRNA modifications have been well characterized[16] enables the prediction of the presence of
133 common modifications, including ms^2i^6A, m^1G, I, and k^2C (Fig 2 and Supplementary Fig. 1). These
134 predictions are strongly supported by the set of tRNA modification enzymes identified in the *Mtb*
135 genome (Fig. 1 and Supplementary Table 3), including *miaA* and *miaB* (ms^2i^6A), *trmD* (m^1G), *tadA* (I),
136 and *tilS* (k^2C). Some tRNA modifications were observed in *Mtb* but are not present in *E. coli*. In other
137 actinobacteria, A58 and A59 are likely modified to m^1A[36], and since *trmI*, the methylase that
138 generates this modification is present in *Mtb*[33], most *Mtb* tRNAs likely contain this modification as
139 well. Nucleoside variation in the sequence of tRNA genes can account for some of the variations in
140 modified sites between *E. coli* and *Mtb*. For example, in *Mtb*, termination signatures derived from G
141 at position 37 were detected in tRNA-Arg2, -Gln1, and -Gln2, whereas this position in these tRNAs
142 in *E. coli* are modified A, such as m^2A and m^6A, which are silent in tRNA-seq. Since m^1G induces
143 strong termination during reverse transcription, these positions are likely modified to m^1G, as
144 observed in the *Bacillus subtilis* tRNA-Arg2 gene position 37 G[37].

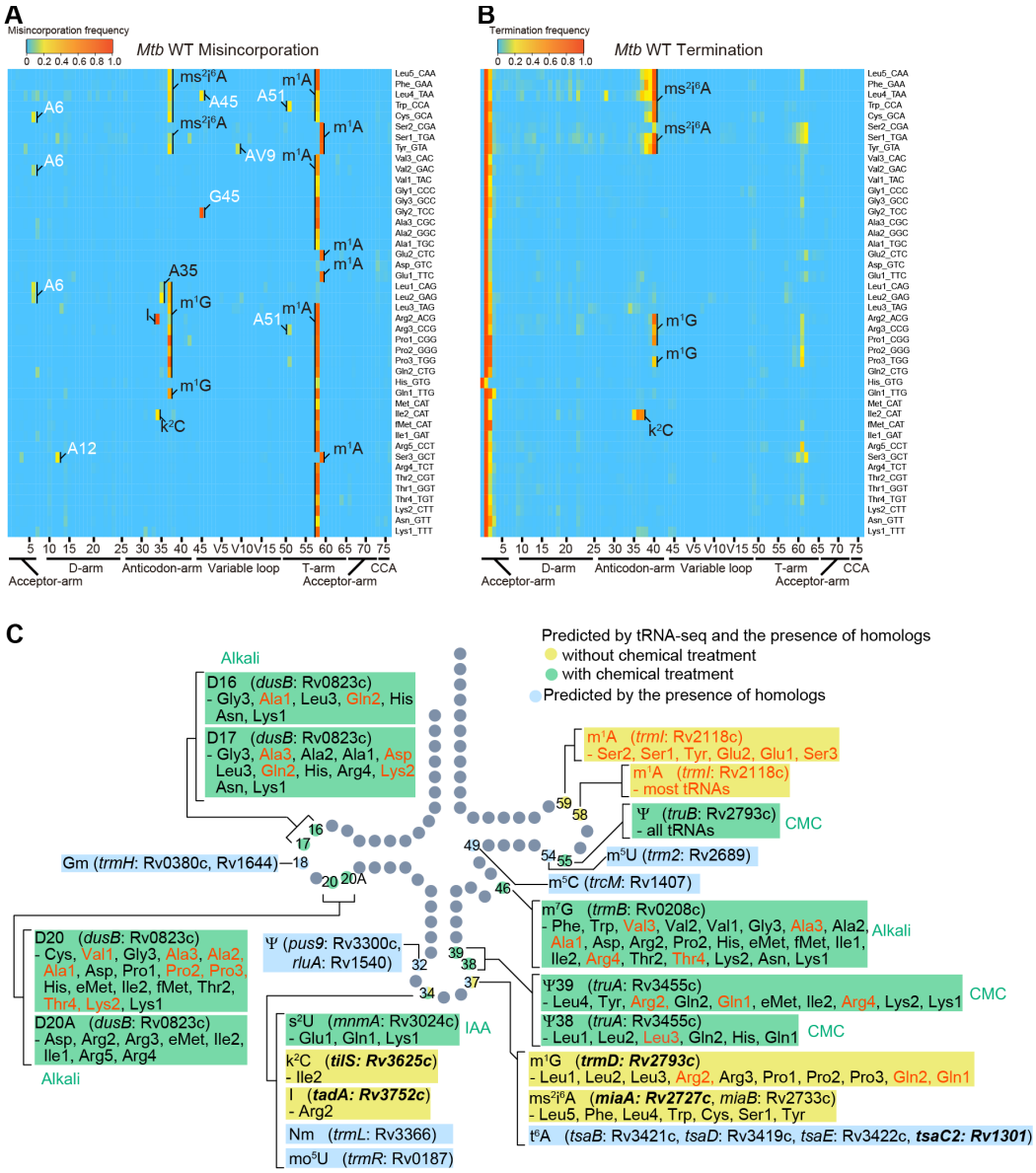

Fig. 2. Heat map of misincorporation and early termination frequency in sequencing of tRNAs from wild type *Mtb*. (A, B) Heatmaps show misincorporation (A) and termination (B) frequencies at all positions across tRNAs (read 5' to 3'). Predicted modifications are labeled based on similarity to known modifications in other organisms and the presence of the tRNA modifying enzyme homologs (Supplementary Table 3). The positions with more than 10% misincorporation in *Mtb* but not in *E. coli* are depicted in white in A. **(C)** *M. tuberculosis* tRNA modifications predicted in this study. Schematic tRNA secondary structure with sites of modifications identified either by the presence of modifying enzymes and/or tRNA-seq. Modifications and tRNA species that are not observed in *E. coli* are shown in red. Modifications and positions that are predicted by both RT-derived signature and the presence of the homologs of tRNA modifying enzymes are shown in yellow (without chemical treatment) and green (with chemical treatment), whereas modifications that are only predicted by the presence of the homologs are shown in light blue. Genes reported to be essential in *Mtb* are shown in bold.

159 tRNA samples were also treated with several chemical treatments prior to sequencing, to
160 expand the set of tRNA modifications detectable by tRNAseq. These treatments included
161 iodoacetamide (IAA), for detection of sulfur modifications,1-cyclohexyl-(2-morpholinoethyl)
162 carbodiimide (CMC) for detection of Ψ, and alkali for detection of D and m^7G. The chemical
163 treatment protocols were first carried out with *E. coli*, to validate the methods.

164 IAA is a thiol-reactive compound that covalently attaches carboxyamidomethyl to thiolated
165 uridines via nucleophilic substitution[38] and modified s^4U is detected as C instead of U. In IAA-
166 treated samples, position 8 and 9, corresponding to s^4U in many tRNA species, had high
167 misincorporation frequencies, confirming that IAA treatment modifies s^4U, leading to elevated
168 misincorporation (Supplementary Fig. 2). Furthermore, we observed higher misincorporation and
169 termination signals at the positions corresponding to other sulfur modification, s^2C, s^2U and their
170 derivatives, such as position 32 in tRNA-Arg3, -Arg5, -Ser3, and Arg4, and 34 in tRNA-Glu, -Gln1,
171 and -Lys (Fig. 3 and Supplementary Fig. 2), revealing that IAA treatment facilitates the detection of
172 not only s^4U but also additional sulfur modifications, which are weakly detected without the IAA
173 treatment.

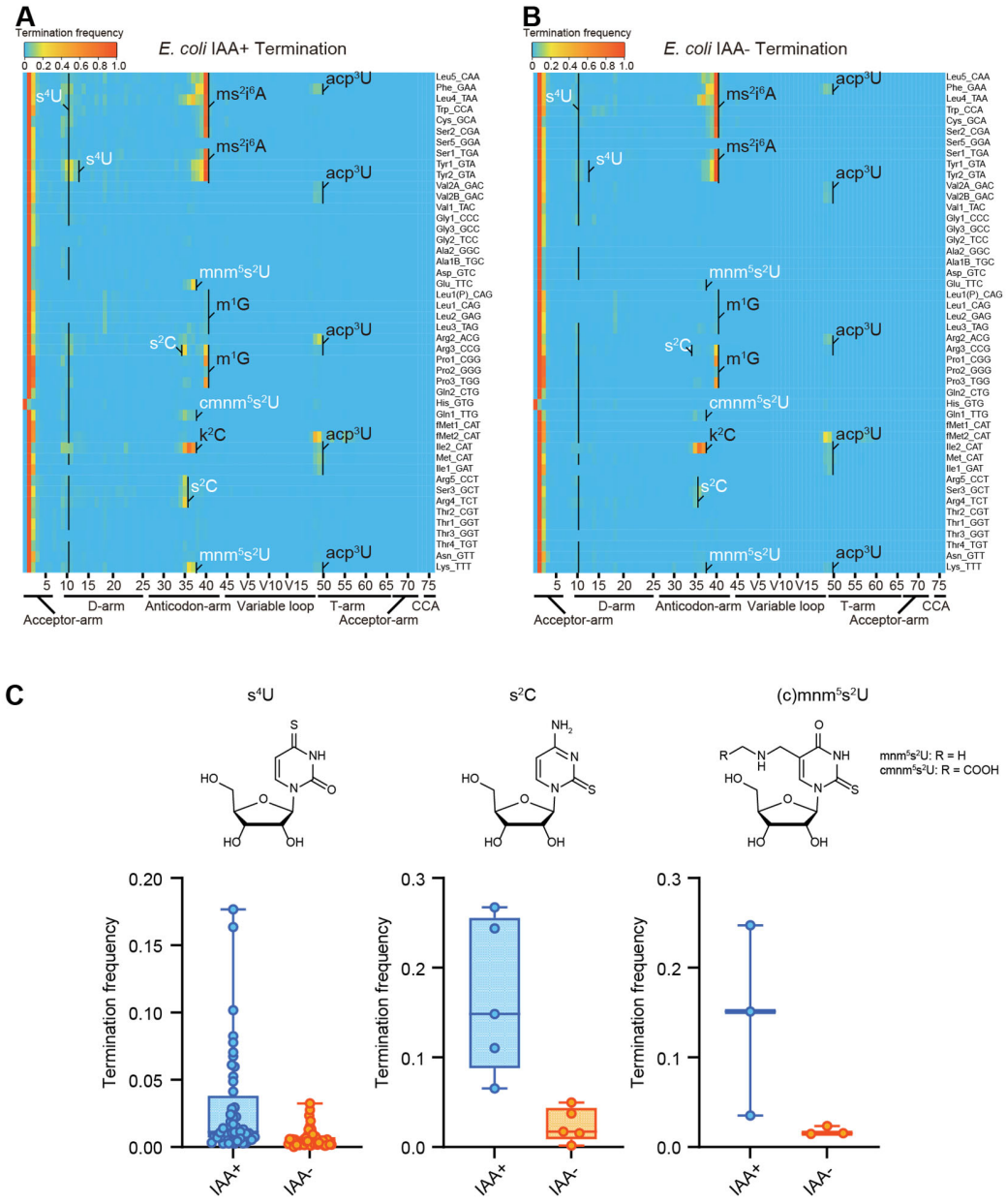

Figure 3. IAA treatment promotes detection of sulfur modifications on tRNAs by enhancing termination signals. (A, B) Heatmaps of the termination signals of *E. coli* tRNAs treated with (A) or without (B) IAA. Known modification sites, including sulfur modifications (s^4U, s^2C, s^2U in white) are shown. (C) Termination frequency at s^4U, s^2C, and s^2U sites of tRNAs treated with or without IAA.

Next, we applied IAA treatment to *Mtb* tRNA-seq. IAA treatment increased termination signals from position 34 in tRNA-Glu1, -Gln1, and -Lys, which contain s^2U derivatives in *E. coli* (Fig. 4A). The 2-thiouridine modification is carried out by MnmA in *E. coli*[39], and a homolog, *Rv3024c*,

183 of this enzyme was identified in the *Mtb* genome (Supplementary Table 3)[40]. We used double
184 stranded DNA-based recombineering to delete Rv3024c in *Mtb,* yielding strain *Mtb⊥mnmA*[41].
185 Sequencing of tRNA isolated from *Mtb⊥mnmA* with prior IAA treatment showed reduced termination
186 signals from position 34 in tRNA-Glu1, -Gln1, and -Lys in treated samples, indicating that Rv3024c
187 plays a critical role in the modification responsible for increased termination frequency derived from
188 position 34 in these tRNAs (Fig. 4B and 4C). Together, these observations strongly suggest that
189 Rv3024c encodes an MnmA-like enzyme that sulfurates position 34 uridines in three *Mtb* tRNA
190 isoacceptors.
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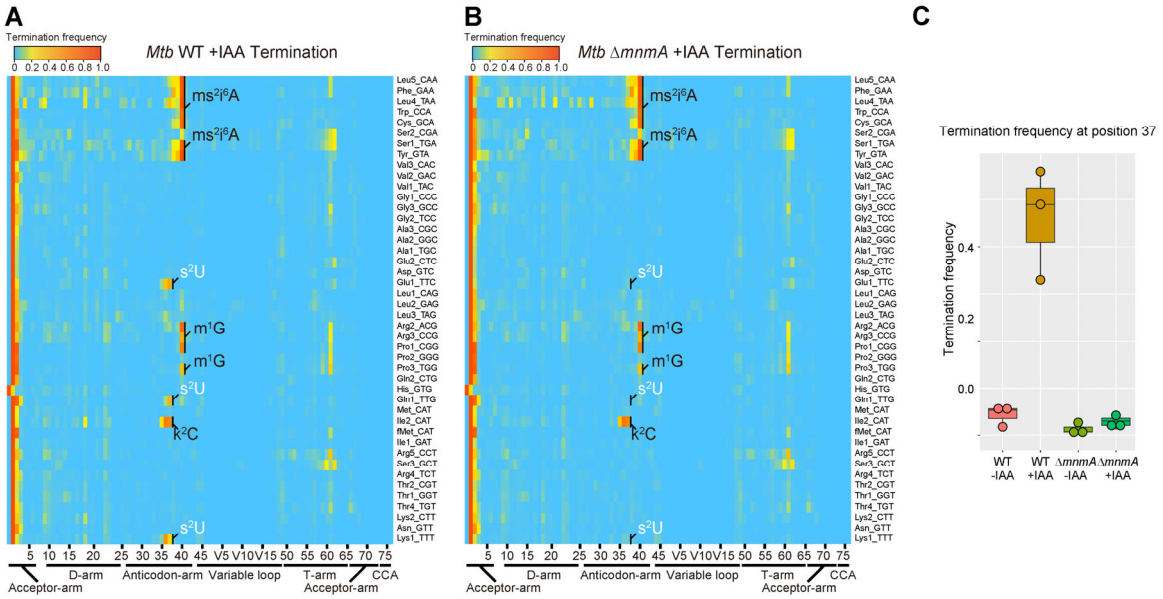

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195 **Fig. 4 Heat map and plot of early termination frequency from sequencing of tRNAs from wild**
196 **type and *MtbΔmnmA* with and without RNA alkylation.** (A, B) Heat map of early termination
197 frequencies across tRNA molecules and positions for WT (A) and *Mtb⊥mnmA* (B). Sulfur
198 modification is shown in white. (C) Plot of termination frequencies at position 37 in wild type (WT)
199 *Mtb* and *Mtb⊥mnmA* for lysine_UUU, glutamate_UUG, and glutamine_UUC isoacceptors. IAA;
200 iodoacetamide.

201 As shown previously[42], CMC treatment increased both misincorporation and termination
202 signatures at a subset of Ψs in *E. coli* tRNAs (Fig 5, and Supplementary Fig. 3 and 4). In addition,
203 CMC-treated samples showed increased frequencies of both misincorporation and termination at
204 position 16, 17, 20, and 20A corresponding to D, and m^7G at position 46. Both modifications are
205 known to undergo base elimination in mild alkali conditions[43]. Since these signals were also
206 observed in the reaction condition in which CMC was not added, these signals are likely attributable
207 to the alkali treatment that is common to both conditions.

208 Reverse transcription-derived signatures derived from alkali-treated D showed a distinctive
209 pattern. With this treatment, when two Ds are at consecutive positions, e.g., D16 and D17, termination
210 signals were elevated at the following position, i.e., position 18 (Supplementary Fig. 3, 5 and 6).
211 Furthermore, alkali treatment also led to higher misincorporation frequencies at singlet Ds (Fig. 5,
212 Supplementary Fig. 5 and 6). Thus, termination and misincorporation signatures enabled the
213 prediction of known *E. coli* tRNA sites modified to D.
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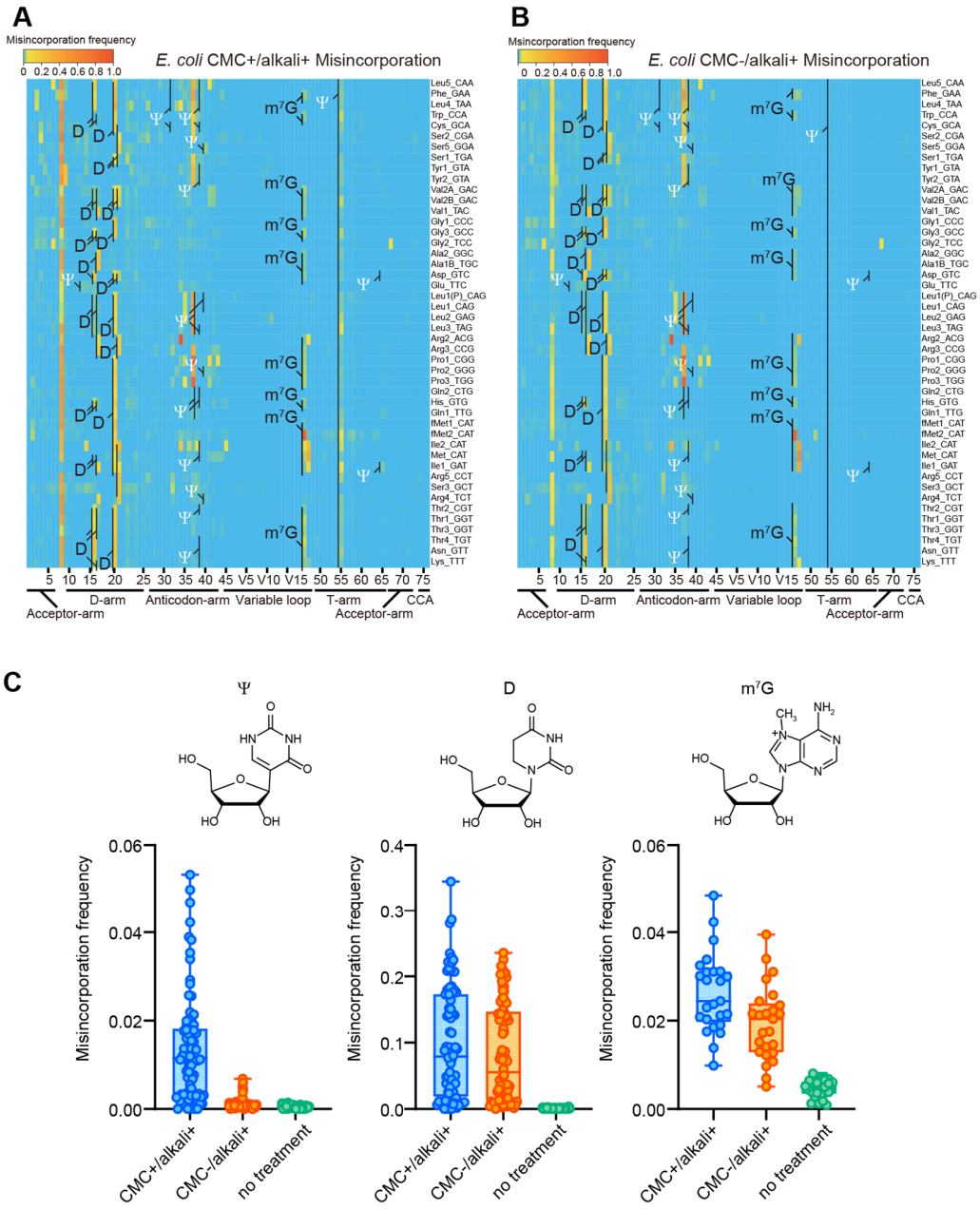

Fig. 5. CMC and alkali treatment facilitate detection of Ψ, D, and m^{7}G modifications in *E. coli*. (A, B) Heatmaps of the misincorporation signals of *E. coli* tRNAs treated with (A) or without (B) CMC. In both conditions, tRNAs are incubated in alkali condition. Known Ψ, D, and m^{7}G sites are shown. Ψ is shown in white. (C) Misincorporation frequency at known Ψ, D, m^{7}G sites of tRNAs treated with CMC+/alkali+ or CMC-/alkali+, and tRNAs without treatment.

224
225 CMC/alkali treatment facilitated the identification of additional modifications in *Mtb* tRNAs.
226 Regardless of CMC treatment, alkali treated samples showed increased misincorporation and
227 termination frequencies derived from U located at position 16, 17, 20, and 20A (Fig. 6 and
228 Supplementary Fig. 7). As observed in *E. coli*, termination signals at position 18 and 21 likely
229 correspond to consecutive Ds at position 16 and 17, and 20 and 20A, respectively. *Mtb* Rv0823c is a
230 homolog of dihydrouridylase DusB (Supplementary Table 3), which likely accounts for the synthesis
231 of D at these positions. Furthermore, alkali treatment increased the misincorporation frequencies at
232 G at position 46 (Supplementary Fig. 8). Since Rv0208c is a homolog of TrmB, which synthesizes
233 m^7G at position 46 in *E. coli*, multiple *Mtb* tRNA species likely contain m^7G at position 46 (Fig. 6 and
234 Supplementary Fig. 8).
235 CMC treatment also increased the termination frequency at several sites. Termination
236 signatures derived from position 55, which is exclusively uridine in all tRNA species, increased in most
237 tRNA species, suggesting that *Mtb* tRNAs contain pseudouridines at this position[37]. Rv2793c is an
238 *Mtb* homologue of *E. coli* TruB and deletion of Rv2793c reduced the termination frequencies at this
239 position in tRNAs isolated from *Mtb*⊥*truB* (Fig. 6 and Supplementary Fig. 9)[41]. Together, these
240 observations suggest that Rv2793c encodes a TruB-like enzyme that modifies position 55 uridines to
241 Ψ across tRNA species. Furthermore, the presence of a TruA homolog in *Mtb* (Rv3455c) suggests
242 that in multiple tRNA species U at positions 38-40 can be modified to Ψ. Indeed, the termination
243 signatures derived from positions 38 and 39 increased depending on CMC treatment, strongly
244 suggesting that these positions are modified to Ψ (Fig. 6 and Supplementary Fig. 9).
245 In total, among 16 tRNA *Mtb* tRNA modifications predicted by the presence of tRNA
246 modifying enzymes (Fig 1 and Supplementary Table 3), 9 species of modifications were detected based
247 on reverse-transcription derived signatures (Fig. 2C).

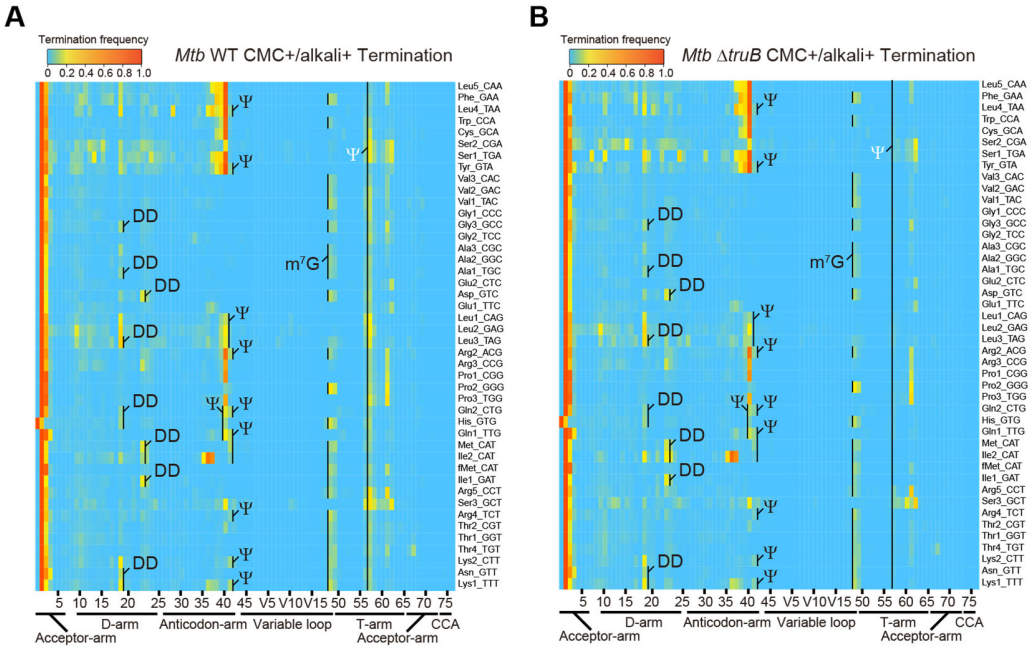

Fig 6. Heat map of early termination frequency from sequencing of tRNAs isolated from WT and *MtbΔtruB* following CMC treatment. Heat map of early termination frequencies across tRNA molecules and positions for WT (left) and *Mtb⊥truB* (right). Termination signals derived from position 55 are shown in white.

Growth of *MtbΔmnmA* is attenuated in a macrophage infection model

To address whether *Mtb* tRNA modifications impact the pathogen's growth in the host environment, we used the MtbTnDB transposon insertion sequencing (Tn-seq) database[44, 45] to determine if transposon insertions in genes encoding tRNA modifying enzymes have been associated with *in vivo* growth defects. Transposon insertions in *mnmA* were reported to attenuate *Mtb* growth in mice infected with a library of *Mtb* transposon mutants, suggesting that *mnmA* facilitates *Mtb* growth *in vivo*. We found that the growth of WT and *Mtb⊥mnmA* were similar in 7H9 medium (Fig. 7), suggesting the absence of s^2U modification at position 34 does not impair *Mtb* growth in culture. In contrast, the *Mtb⊥mnmA* mutant was significantly impaired for growth in a macrophage infection model[45] (Fig. 7). Defective growth of the *Mtb⊥mnmA* mutant was also observed in macrophages treated with all-trans retinoic acid (ATRA), which promotes macrophage control of *Mtb* infection[46].

265 These observations strongly suggest that modification of U to s^2U by MnmA facilitates *Mtb* growth in

266 macrophages.

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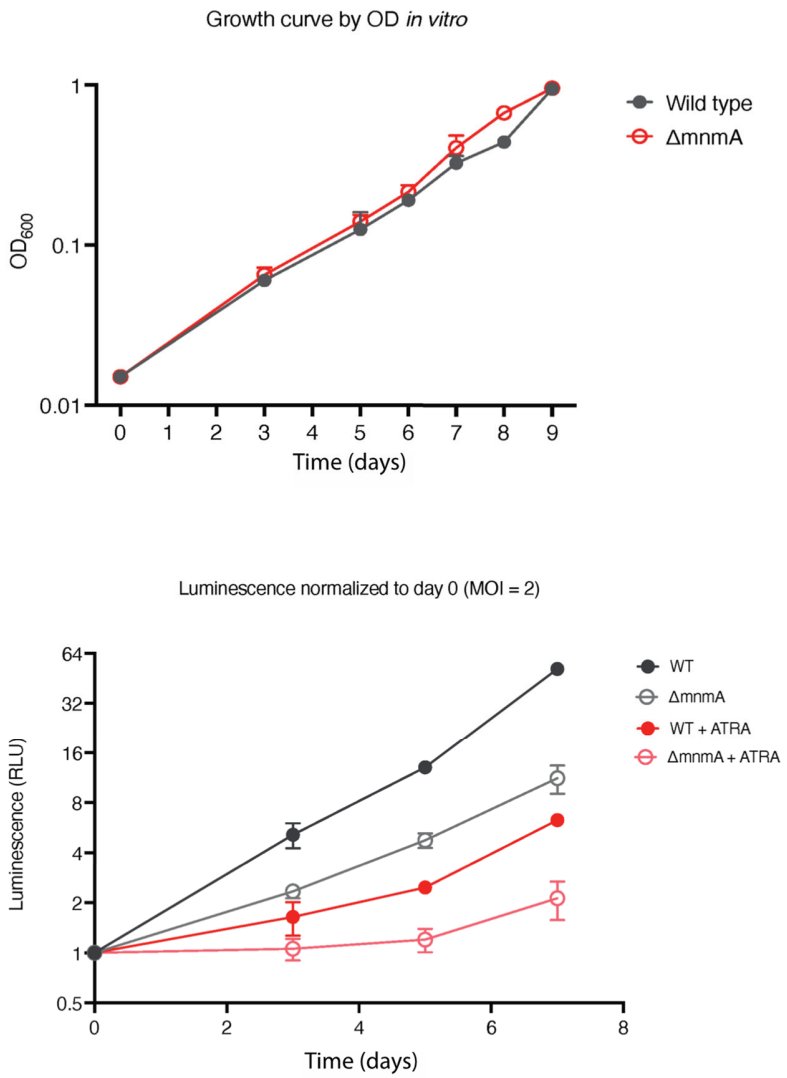

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269 **Fig. 7. *MtbΔmnmA* is attenuated in a macrophage infection model**. Top: Wild type and
270 *MtbΔmnmA* do not display growth differences in 7H9 medium. Bottom: Auto-luminescent wild type
271 and *ΔmnmA Mtb* strains were diluted to a multiplicity of infection of 2 bacteria per mouse bone
272 marrow-derived macrophage with or without all-trans retinoic acid (ATRA). Survival was measured
273 by luminescence and normalized to luminescence at time 0.
274

275

276 **Discussion**

277 Here, we profiled *Mtb* tRNA modifications with tRNA sequencing to provide the first maps
278 of the tRNA modification landscape in this global pathogen. In total, nine modifications, including
279 six modifications without chemical treatment, were identified based on reverse transcription derived
280 signatures. CMC/alkali treatment and IAA treatment further identified Ψ and m^7G and sulfur
281 modifications, respectively. Although we did not chemically validate the modifications predicted by
282 tRNA-seq with mass spectrometry, the identification of *Mtb* homologs of tRNA modifying enzymes
283 strongly bolsters the RT-signature-based predictions. Furthermore, the deletion of *truB* and *mnmA*
284 genes in *Mtb* eliminated the respective modification signatures of pseudouridine and s^2U, validating
285 that the enzymes encoded by these genes synthesize these modifications. Finally, the growth defect of
286 the Δ*mnmA* strain within macrophages but not in a nutrient-rich medium suggests that s^2U tRNA
287 modification facilitates *Mtb* adaptation to the host intracellular environment.

288 IAA treatment was developed for detecting s^4U modification in pulse-chase experiments to
289 measure RNA turnover[38]. We found that this treatment enhances the RT-signatures of additional
290 sulfur modifications as well, including s^2C and s^2U, indicating that IAA should have general utility in
291 tRNA-seq-based profiling of sulfur modifications, including in studies tracking changes in tRNA
292 sulfuration[47, 48].

293 Multiple modifications do not cause reverse transcriptase errors. Although tRNA-seq provides
294 a simple and rapid method for profiling the landscape of modifications in all tRNA species, this
295 approach does not enable comprehensive identification of modifications. Here, several modifications,
296 including t^6A, m^5U, mo^5U, m^5C, Cm, Um, and Gm, predicted by the presence of *Mtb* homolog of
297 tRNA modifying enzymes, such as TsaC2, TsaB, TsaD, TsaE, Trm2, TrcM, TrmR, TrmL, and TrmH,
298 were not detected in tRNA-seq. Additionally, CMC treatment may not determine all Ψ positions, thus,
299 the target of Rv3300 and Rv1540 remain unclear. Since these genes are similar to *E. coli rluA,* which

300 also targets rRNA, these genes may target rRNAs instead of tRNAs. Mapping and further analysis of
301 these modifications will require different approaches such as tRNA purification and RNA mass
302 spectrometric analysis.

303 Several strong RT signatures were detected in *Mtb* tRNAs but not in *E. coli* (Fig. 2A), including
304 G45 in tRNA-Gly2. Most of these signals are strict misincorporations of C or T at A or G position,
305 respectively. Since modifications at a purine position, e.g., m^1G and m^1A, generally cause random
306 misincorporation of the other three nucleosides, these *Mtb*-specific misincorporation signatures are
307 not likely to be derived from modification. Further mass spectrometric analysis of purified tRNAs will
308 be necessary to assess whether these signals are indeed derived from *Mtb*-specific modifications; such
309 *Mtb*-specific modifications could be related to *Mtb* pathogenesis and/or survival in the host.

310 The role of s^2U in *Mtb* appears to be unusual. s^2U is a universally conserved modification
311 observed in all three domains of life at the wobble position in the anticodon. This modification
312 enhances the stacking of s^2U with U35 to stabilize the anticodon structure, facilitating codon-
313 anticodon interactions. s^2U is also recognized by multiple aminoacyl-tRNA synthetases for efficient
314 amino acylation[12]. Although elimination of this sulfur modification causes severe growth
315 phenotypes in most organisms[39, 49], unexpectedly, the deletion of *mnmA* in *Mtb* did not attenuate
316 growth of the pathogen *in vitro*, suggesting that the *Mtb* requirement for s^2U modification differs from
317 other organisms. The marked growth retardation of Δ*mnmA* strain within macrophages indicates the
318 specific requirement of this modification within host cells. This modification may be necessary for
319 maintaining general translation efficiency inside host cells and/or facilitate the expression of specific
320 genes that are necessary for survival within macrophages. In fact, lack of *mnmA* is reported to sensitize
321 *E. coli* to oxidative stress, raising the possibility that s^2U modification promotes *Mtb* growth under
322 oxidative stress elicited by the host.

323 Differential codon usage between house-keeping genes and virulence genes could contribute
324 to the differential growth phenotypes observed *in vitro* and *in vivo* in the Δ*mnmA* mutant. Among
325 multiple codons encoding Glu, Gln, and Lys, s^2U modification dependent codon usage might be
326 preferentially distributed in genes associated with intracellular growth. For example, *Mtb* has two
327 tRNA isoacceptors, tRNA-Glu1(s^2UUC) and tRNA-Glu2(CUC), to decipher two Glu codons, GAA
328 and GAG. According to the wobble paring rule, GAA is only decoded by tRNA-Glu1(s^2UUC),
329 whereas GAG is decoded by both tRNA-Glu1(s^2UUC) and tRNA-Glu2(CUC); i.e., GAG can be
330 deciphered by an s^2U-independent tRNA. Thus, genes required for intracellular growth might be
331 enriched with GAA, an s^2U-dependent codon. Similar codon usage differences could be present in
332 Gln and Lys codons deciphered by s^2U-containing tRNAs.

333 In most organisms, s^2U is further modified into derivatives containing an additional chemical
334 moiety at position 5[2, 50, 51]. However, *Mtb* does not contain apparent homologs of the tRNA
335 modifying enzymes that introduce the additional modifications to s^2U. Thus, *Mtb* may contain s^2U or
336 s^2U derivatives synthesized by other types of enzymes. Additional analyses to elucidate the structures
337 of modified s^2U in *Mtb* are warranted.

338 Another unexpected finding is the presence of a TrmR homolog in *Mtb*. TrmR in *Bacillus*
339 *subtilis* is a methylase that converts a 5-hydroxyuridine (ho^5U) into a 5-methoxyuridine (mo^5U) at the
340 tRNA wobble position, suggesting that *Mtb* possesses mo^5U. However, *Mtb* does not encode an
341 apparent homolog for the enzyme that mediates the RNA hydroxylation reaction that yields ho^5U, the
342 substrate for TrmR. Although we cannot rule out the possibility that *Mtb* TrmR methylates a substrate
343 other than RNA, it is possible that *Mtb* utilizes an unknown hydroxylation pathway for synthesizing
344 ho^5U.

345 Our findings serve as a valuable starting point for the research community to continue
346 characterizing the physiological roles and mechanisms of *Mtb* tRNA modifications. Since tRNA-seq

347 offers an efficient and scalable platform for surveying changes in tRNA modifications, this approach
348 will be valuable across growth conditions, and may be extended to growth inside host cells. Finally,
349 further studies elucidating the mechanisms by which tRNA modifications facilitate *Mtb* growth in host
350 cells should be valuable for designing new therapeutics for tuberculosis.
351
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357
358

359 **Materials and Methods**

360 **Bacterial strains and growth conditions:** *Mtb* strains were grown from frozen stocks into
361 Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween-80, and ADC (5 g/L
362 bovine serum albumin, 2 g/L dextrose, 3 μg/ml catalase). Cultures were incubated at 37 °C. Strains
363 were grown to mid log-phase for all experiments (OD_{600} 0.4-0.6). Growth was measured on a BioTek
364 plate reader for *in vitro* growth by measuring OD_{600} every 24 hours.

365

366 **Bacterial strain construction:** Supplementary Table 4 depicts the strains, plasmids, primers, and
367 recombinant DNA used for this study. Plasmids were built by restriction digest of a parental vector
368 and inserts were prepared by Gibson assembly[52]. Plasmids were isolated from *E. coli* and confirmed
369 via Sanger sequencing carried out by Genewiz, LLC (Massachusetts, USA).

370

371 Deletion mutants: The knockout strain *Mtb*Δ*mnmA*::zeo (zeocin) was built using double-stranded
372 recombineering in the parental *Mtb* strain H37Rv. A linear dsDNA fragment was constructed using
373 stitch PCR with the primers listed in Supplementary Table 4 which consisted of a 500bp region
374 upstream of *mnmA* (Rv3024c)), 500 bp downstream region, and a *lox*-zeo-*lox* fragment. This cassette
375 was transformed into an H37Rv recombineering strain as described[53] and plated on 7H10 + zeocin
376 plates.

377

378 **Homology search**

379 Local BLAST was performed to search for *Mtb* homologs of tRNA modifying enzymes. First, the
380 uniport IDs of tRNA modifying enzymes were obtained from Modomics[24], and 12 proteins were
381 manually added to the list [16,54–58], including Q47319/TapT, P24188/TrhO, P76403/TrhP,
382 O32034/TrhP1, O32035/TrhP2, P36566/CmoM, and Q87K36/TrcP, O34614/MnmM,

383 Q8N5C7/DTWD1, Q8NBA8/DTWD2, O32036/TrmR, Q9KV41/AcpA. Uniprot ID provides a
384 fasta file of tRNA modifying enzymes from the Uniprot database[59]. A blast database file was
385 generated by 'makeblastdb' script using a fasta file of *Mtb* proteins (H37Rv strain) retrieved from
386 NCBI. Then, the homologs of tRNA modifying enzymes were searched against the *Mtb* protein
387 database using the fasta file of tRNA modifying enzymes as a query. Output format is defined by the
388 following script: -outfmt "7 qacc sacc stitle score qcovs evalue pident" -evalue 1e-10. The output file
389 was modified by excel (Supplementary Table 4).
390

391 **Phylogenetic analysis of *Mtb* tRNA modifying enzyme homologs**

392 Local BLAST was conducted to search for homologs of *Mtb* tRNA modifying enzymes in 120
393 manually picked organisms across three domains of life. A custom database was generated by
394 combining the fasta files of organisms' proteins retrieved from NCBI. Homologs of eighteen *Mtb*
395 tRNA modifying enzymes were searched against the custom protein database using local blast.
396 Log10Evalues were visualized by iTol[34] with a phylogenetic tree generated by phyloT[60].
397

398 **tRNA sequencing**

399 Extraction of total RNA: Strains were grown to mid-log phase with the appropriate antibiotics and
400 inducing agents described above. RNA was collected at the same OD_{600} for each strain (between 0.4-
401 0.6). Cells were left on ice for 20 minutes, then pelleted by centrifuging at 4,000 rpm for 10 minutes
402 at 4 °C. Pellets were resuspended in 0.5-1 mL of TriZol (Life Technologies) and lysed using a BeadBug
403 microtube homogenizer (Millipore Sigma). 200 μL of chloroform was added to each tube, after which
404 samples obtained from *Mtb* strains were removed from biosafety level 3 precautions. Samples were
405 centrifuged at 15,000 rpm for 15 minutes at 4 °C and the aqueous layer was collected into a fresh tube.
406 To the original tube, 250 μL of sodium acetate buffer (300 mM sodium acetate pH 5.2 and 10 mM

407 EDTA pH 8.0) was added, and samples were vortexed at 4 °C for 5 minutes then centrifuged at 15,000
408 g for 15 minutes at 4 °C. The aqueous layer was added to the fresh sample-containing tubes. 400 μL
409 chloroform was added, and tubes were briefly vortexed and then centrifuged at 15,000 rpm for 1
410 minute at 4 °C. The aqueous phase was collected into a fresh tube and RNA recovered by ethanol
411 precipitation. RNA pellets were resuspended in 10 mM sodium acetate pH 5.2 and stored at -80 °C
412 until processed for sequencing. Total RNA samples were alkali-treated prior to tRNA extraction to
413 deacylate all tRNAs (1 hour at 37 °C in 100 mM Tris-HCl pH 9.0).
414
415 Isolation of tRNA fraction: 1–2 μg of total RNA was run on a 10% TBE-UREA gel (ThermoFisher
416 Scientific) at 250 V for 1 hour. Gels were stained with SYBR Gold (ThermoFisher Scientific), and
417 tRNA was excised. Excised gels containing tRNA fractions were mashed in RNAse-free tubes, and
418 300 μL elution buffer (300 mM NaOAc pH 5.5, 1 mM EDTA pH 8.0, 0.10% SDS) was added to each
419 tube. Samples were shaken on a thermoshaker (Eppendorf) for 1–4 hours at 37 °C and supernatant
420 was collected using an Ultrafree filter column (Millipore Sigma). tRNA was recovered by isopropanol
421 precipitation.
422
423 tRNA dephosphorylation: tRNA was dephosphorylated using QuickCIP (New England BioLabs)
424 according to manufacturer instructions, and tRNA was collected by phenol-chloroform extraction
425 followed by isopropanol precipitation.
426
427 Iodoacetamide (IAA) treatment: IAA treatment was performed as described[38]. Briefly, 500 ng of
428 total RNA is combined with 10 mM of iodoacetamide, 50 mM $NaPO_4$ pH 8.0, and 50% DMSO in a
429 final volume of 50 μL. Reactions were incubated at 50 °C for 15 minutes and quenched with DTT.

430

431 CMC treatment: CMC treatment was carried out as described in ref. Briefly, 2.5 μg tRNA fraction in
432 0.5 μl was mixed with 15 μl CMC-BEU buffer with or without CMC (0.34 M or 0 M CMC, 7 M urea,
433 4 mM EDTA, and 50 mM bicine pH 7.9) and incubated at 37 °C for 20 min. Adding 100 μl CMC
434 stop solution (0.3 M NaOAc pH 5.2 and 100 mM EDTA) quenched the reaction. RNA was desalted
435 with PD-10 desalting column (Cytiva) and recovered by ethanol precipitation. RNA was dissolved in
436 40 μl of 50 mM sodium carbonate buffer (pH 10.4) and incubated at 37 °C for 4 hours, followed by
437 ethanol precipitation.

438

439 Adapter ligation: 0.5 μL RNase inhibitor was added to 3.5μL dephosphorylated tRNA (200–250 ng
440 tRNA) and samples were boiled at 80 °C for 2 minutes. Boiled tRNA was mixed with 12 μL PEG
441 buffer mix (10 μL 50% PEG8000, 2 μL 10 × buffer B0216S; New England Biolabs). 3 μL of 5'
442 adenylated linkers (Supplementary Table 4) were added (33 pmol/μL) along with 1 μL T4 RNA ligase
443 2 truncated (New England BioLabs) and incubated at 25 °C for 2.5 hours. Samples were recovered by
444 isopropanol precipitation and run on a 10% TBE-Urea PAGE gel for 40 minutes at 250 V. Ligated
445 products were recovered by gel excision as described above.

446

447 Reverse transcription: Identical quantities of samples with different adapter sequences were pooled
448 for reverse transcription for a total of 200–250 ng tRNA. Reverse transcription was performed by
449 combining 2.1 μL dephosphorylated tRNA with 100 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1.25 μM
450 RT primer (Supplementary Table 4), 450 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 500 nM TGIRT
451 (InGex), and 15% PEG8000 in a final volume of 9 μL. Samples were incubated at 25 °C for 30
452 minutes, after which 1 μL 10 mM dNTPs (New England BioLabs) were added and reactions incubated

453 at 60 °C for 1 hour. 1.15 μL NaOH was added, and samples were boiled for 15 minutes and run on a
454 10% TBE Urea PAGE gel at 250V for 1 hour. Reverse transcription products were excised from gels
455 and cDNA recovered by isopropanol precipitation. Linear single-stranded cDNA was circularized
456 using CircLigase II (Lucigen) in accordance with manufacturer instructions.
457
458 PCR of tRNA libraries: PCR reactions were set up using HF Phusion according to the manufacturer's
459 instructions using a universal reverse primer (Supplementary Table 4) and a different index primer for
460 each pool of samples. PCR reactions were aliquoted into 4 tubes and collected after 6, 8, 10, and 12
461 cycles. Samples were run on a Native TBE PAGE gel (ThermoFisher Scientific) at 180 V for 50
462 minutes, and amplified products were cut from the same cycle for each sequencing run. Samples were
463 recovered by gel excision and isopropanol precipitation.
464
465 Sequencing: Sequencing was performed on a MiSeq instrument (Illumina) using 150 bp single end
466 reads with a version 3, 150 cycle kit.
467
468 Analysis: 3' linker sequences and two nucleotides at the 5' end were trimmed using cutadapt and fastx-
469 trimmer. Bowtie v1.2.2 was used with default settings to map reads to reference *Mtb* tRNA sequences
470 retrieved from Mycobrowser[40] (Supplementary Table 5). Mpileup files were generated using
471 samtools (samtools mpileup -I -A --ff 4 -x -B -q 0 -d 10000000). For analysis of termination
472 frequencies, 5' end termini of mapped reads were piled up using bedtools genomecov (option, -d -5 -
473 ibam). The number of 5' termini at each tRNA position was divided by the total number of mapped
474 termini at that position plus all upstream (5') positions.
475

476 Macrophage infection: Auto-luminescent wild type and ⊿*mnmA Mtb* strains grown to the same OD_{600}
477 were pelleted by centrifugation and prepared in RPMI media by soft spinning as described[61]. Briefly,
478 cells were washed, pelleted, resuspended, and centrifuged at 121 g, with the top half of the centrifuged
479 supernatant used. Suspensions were diluted to a multiplicity of infection of 2 bacteria per mouse bone
480 marrow-derived macrophage by determining the OD_{600}. Macrophages were infected for 6 hours,
481 followed by a PBS wash and addition of RPMI with or without all-trans retinoic acid (ATRA). ATRA
482 promotes macrophage control of *Mtb* infection[46] and was used to assess strain survival in an
483 increasingly restricted macrophage environment. Survival was measured by luminescence in a BioTek
484 plate reader and normalized to luminescence reads at time 0.
485

References

1. Huang, H.Y. and A.K. Hopper, *Multiple Layers of Stress-Induced Regulation in tRNA Biology.* Life (Basel), 2016. **6**(2).
2. Björk, G.R. and T.G. Hagervall, *Transfer RNA Modification: Presence, Synthesis, and Function.* EcoSal Plus, 2014. **6**(1).
3. Shepherd, J. and M. Ibba, *Bacterial transfer RNAs.* FEMS Microbiol Rev, 2015. **39**(3): p. 280-300.
4. Torrent, M., et al., *Cells alter their tRNA abundance to selectively regulate protein synthesis during stress conditions.* Sci Signal, 2018. **11**(546).
5. Orellana, E.A., E. Siegal, and R.I. Gregory, *tRNA dysregulation and disease.* Nat Rev Genet, 2022. **23**(11): p. 651-664.
6. Suzuki, T., *The expanding world of tRNA modifications and their disease relevance.* Nat Rev Mol Cell Biol, 2021. **22**(6): p. 375-392.
7. Liu, Y., et al., *tRNA-m(1)A modification promotes T cell expansion via efficient MYC protein synthesis.* Nat Immunol, 2022. **23**(10): p. 1433-1444.
8. Goodarzi, H., et al., *Modulated Expression of Specific tRNAs Drives Gene Expression and Cancer Progression.* Cell, 2016. **165**(6): p. 1416-1427.
9. Delaunay, S., et al., *Mitochondrial RNA modifications shape metabolic plasticity in metastasis.* Nature, 2022. **607**(7919): p. 593-603.
10. Nedialkova, D.D. and S.A. Leidel, *Optimization of Codon Translation Rates via tRNA Modifications Maintains Proteome Integrity.* Cell, 2015. **161**(7): p. 1606-18.
11. Kimura, S. and M.K. Waldor, *The RNA degradosome promotes tRNA quality control through clearance of hypomodified tRNA.* Proc Natl Acad Sci U S A, 2019. **116**(4): p. 1394-1403.
12. Giegé, R. and G. Eriani, *The tRNA identity landscape for aminoacylation and beyond.* Nucleic Acids Res, 2023.
13. de Crécy-Lagard, V. and M. Jaroch, *Functions of Bacterial tRNA Modifications: From Ubiquity to Diversity.* Trends Microbiol, 2021. **29**(1): p. 41-53.
14. Zhang, W., et al., *tRNA modification dynamics from individual organisms to metaepitranscriptomics of microbiomes.* Mol Cell, 2022. **82**(5): p. 891-906.
15. Zheng, G., et al., *Efficient and quantitative high-throughput tRNA sequencing.* Nat Methods, 2015. **12**(9): p. 835-837.
16. Kimura, S., P.C. Dedon, and M.K. Waldor, *Comparative tRNA sequencing and RNA mass spectrometry for surveying tRNA modifications.* Nat Chem Biol, 2020. **16**(9): p. 964-972.
17. Motorin, Y. and M. Helm, *Methods for RNA Modification Mapping Using Deep Sequencing: Established and New Emerging Technologies.* Genes (Basel), 2019. **10**(1).
18. Finet, O., et al., *Transcription-wide mapping of dihydrouridine reveals that mRNA dihydrouridylation is required for meiotic chromosome segregation.* Mol Cell, 2022. **82**(2): p. 404-419 e9.
19. Draycott, A.S., et al., *Transcriptome-wide mapping reveals a diverse dihydrouridine landscape including mRNA.* PLoS Biol, 2022. **20**(5): p. e3001622.
20. Dai, Q., et al., *Quantitative sequencing using BID-seq uncovers abundant pseudouridines in mammalian mRNA at base resolution.* Nat Biotechnol, 2022.
21. W.H.O., *Global tuberculosis report 2021.* 2021.

532 22. Chionh, Y.H., et al., *tRNA-mediated codon-biased translation in mycobacterial hypoxic*
533 *persistence.* Nat Commun, 2016. **7**: p. 13302.
534 23. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-
535 10.
536 24. Boccaletto, P., et al., *MODOMICS: a database of RNA modification pathways. 2021*
537 *update.* Nucleic Acids Res, 2022. **50**(D1): p. D231-D235.
538 25. DeJesus, M.A., et al., *Comprehensive Essentiality Analysis of the Mycobacterium*
539 *tuberculosis Genome via Saturating Transposon Mutagenesis.* mBio, 2017. **8**(1).
540 26. Bosch, B., et al., *Genome-wide gene expression tuning reveals diverse vulnerabilities of*
541 *M. tuberculosis.* Cell, 2021. **184**(17): p. 4579-4592 e24.
542 27. Masuda, I., et al., *tRNA methylation resolves codon usage bias at the limit of cell*
543 *viability.* Cell Rep, 2022. **41**(4): p. 111539. E.
544 28. Soma, A., et al., *An RNA-modifying enzyme that governs both the codon and amino acid*
545 *specificities of isoleucine tRNA.* Mol Cell, 2003. **12**(3): p. 689-98.
546 29. Wolf, J., A.P. Gerber, and W. Keller, *tadA, an essential tRNA-specific adenosine*
547 *deaminase from Escherichia coli.* EMBO J, 2002. **21**(14): p. 3841-51.
548 30. El Yacoubi, B., et al., *The universal YrdC/Sua5 family is required for the formation of*
549 *threonylcarbamoyladenosine in tRNA.* Nucleic Acids Res, 2009. **37**(9): p. 2894-909.
550 31. Anderson, J., et al., *The essential Gcd10p-Gcd14p nuclear complex is required for 1-*
551 *methyladenosine modification and maturation of initiator methionyl-tRNA.* Genes Dev,
552 1998. **12**(23): p. 3650-62.
553 32. Guelorget, A., et al., *Insights into the hyperthermostability and unusual region-specificity*
554 *of archaeal Pyrococcus abyssi tRNA m1A57/58 methyltransferase.* Nucleic Acids Res,
555 2010. **38**(18): p. 6206-18.
556 33. Varshney, U., et al., *Mycobacterium tuberculosis Rv2118c codes for a single-component*
557 *homotetrameric m1A58 tRNA methyltransferase.* Nucleic Acids Res, 2004. **32**(3): p.
558 1018-27.
559 34. Letunic, I. and P. Bork, *Interactive Tree Of Life (iTOL) v5: an online tool for*
560 *phylogenetic tree display and annotation.* Nucleic Acids Res, 2021. **49**(W1): p. W293-
561 W296.
562 35. Kellner, S., J. Burhenne, and M. Helm, *Detection of RNA modifications.* RNA Biol, 2010.
563 **7**(2): p. 237-47.
564 36. Schwartz, M.H., et al., *Microbiome characterization by high-throughput transfer RNA*
565 *sequencing and modification analysis.* Nat Commun, 2018. **9**(1): p. 5353.
566 37. Juhling, F., et al., *tRNAdb 2009: compilation of tRNA sequences and tRNA genes.*
567 Nucleic Acids Res, 2009. **37**(Database issue): p. D159-62.
568 38. Herzog, V.A., et al., *Thiol-linked alkylation of RNA to assess expression dynamics.* Nat
569 Methods, 2017. **14**(12): p. 1198-1204.
570 39. Kambampati, R. and C.T. Lauhon, *MnmA and IscS are required for in vitro 2-thiouridine*
571 *biosynthesis in Escherichia coli.* Biochemistry, 2003. **42**(4): p. 1109-17.
572 40. Kapopoulou, A., J.M. Lew, and S.T. Cole, *The MycoBrowser portal: a comprehensive*
573 *and manually annotated resource for mycobacterial genomes.* Tuberculosis (Edinb),
574 2011. **91**(1): p. 8-13.
575 41. van Kessel, J.C. and G.F. Hatfull, *Recombineering in Mycobacterium tuberculosis.* Nat
576 Methods, 2007. **4**(2): p. 147-52.

42. Carlile, T.M., et al., *Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells.* Nature, 2014. **515**(7525): p. 143-6.
43. Marchand, V., et al., *Mapping of 7-methylguanosine (m(7)G), 3-methylcytidine (m(3)C), dihydrouridine (D) and 5-hydroxycytidine (ho(5)C) RNA modifications by AlkAniline-Seq.* Methods Enzymol, 2021. **658**: p. 25-47.
44. Zhang, Y.J., et al., *Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing.* Cell, 2013. **155**(6): p. 1296-308.
45. Jinich, A., et al., *The Mycobacterium tuberculosis transposon sequencing database (MtbTnDB): a large-scale guide to genetic conditional essentiality.* bioRxiv, 2021.
46. Babunovic, G.H., et al., *CRISPR Interference Reveals That All-Trans-Retinoic Acid Promotes Macrophage Control of Mycobacterium tuberculosis by Limiting Bacterial Access to Cholesterol and Propionyl Coenzyme A.* mBio, 2022. **13**(1): p. e0368321.
47. Edwards, A.M., K.A. Black, and P.C. Dos Santos, *Sulfur Availability Impacts Accumulation of the 2-Thiouridine tRNA Modification in Bacillus subtilis.* J Bacteriol, 2022. **204**(5): p. e0000922.
48. Laxman, S., et al., *Sulfur amino acids regulate translational capacity and metabolic homeostasis through modulation of tRNA thiolation.* Cell, 2013. **154**(2): p. 416-29.
49. Dewez, M., et al., *The conserved Wobble uridine tRNA thiolase Ctu1-Ctu2 is required to maintain genome integrity.* Proc Natl Acad Sci U S A, 2008. **105**(14): p. 5459-64.
50. Karlsborn, T., et al., *Elongator, a conserved complex required for wobble uridine modifications in eukaryotes.* RNA Biol, 2014. **11**(12): p. 1519-28.
51. Asano, K., et al., *Metabolic and chemical regulation of tRNA modification associated with taurine deficiency and human disease.* Nucleic Acids Res, 2018. **46**(4): p. 1565-1583.
52. Gibson, D.G., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases.* Nat Methods, 2009. **6**(5): p. 343-5.
53. Murphy, K.C., K. Papavinasasundaram, and C.M. Sassetti, *Mycobacterial recombineering.* Methods Mol Biol, 2015. **1285**: p. 177-99.
54. Takakura, M., et al., *Biogenesis and functions of aminocarboxypropyluridine in tRNA.* Nat Commun, 2019. **10**(1): p. 5542.
55. Cho, G., J. Lee, and J. Kim, *Identification of a novel 5-aminomethyl-2-thiouridine methyltransferase in tRNA modification.* Nucleic Acids Res, 2023. **51**(4): p. 1971-1983.
56. Sakai, Y., S. Kimura, and T. Suzuki, *Dual pathways of tRNA hydroxylation ensure efficient translation by expanding decoding capability.* Nat Commun, 2019. **10**(1): p. 2858.
57. Sakai, Y., et al., *Biogenesis and growth phase-dependent alteration of 5-methoxycarbonylmethoxyuridine in tRNA anticodons.* Nucleic Acids Res, 2016. **44**(2): p. 509-23.
58. Kimura, S., et al., *Sequential action of a tRNA base editor in conversion of cytidine to pseudouridine.* Nat Commun, 2022. **13**(1): p. 5994.
59. UniProt, C., *UniProt: the Universal Protein Knowledgebase in 2023.* Nucleic Acids Res, 2023. **51**(D1): p. D523-D531.
60. *PhyloT: a tree generator.*
61. Saito, K., et al., *Rifamycin action on RNA polymerase in antibiotic-tolerant Mycobacterium tuberculosis results in differentially detectable populations.* Proc Natl Acad Sci U S A, 2017. **114**(24): p. E4832-E4840.