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 18 candidate tRNA modifying enzymes that are predicted to create 13 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.

28 **Introduction**

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

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

46 tRNA sequencing (tRNA-seq) allows for the rapid and systematic prediction of many tRNA
47 modification sites[14, 15]. We recently developed a comparative tRNA-seq protocol to profile tRNA
48 modifications in organisms with uncharted tRNA modification profiles; in *Vibrio cholerae,* this
49 approach led to the discovery of a new RNA modification and RNA editing process[16]. tRNA-seq
50 enables rapid prediction of modified sites through detection of reverse transcription-derived
51 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 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]. With a stringent threshold (E-value $<1\times10^{-10}$), 20 *Mtb* genes homologous to
77 genes encoding known RNA modification enzymes were identified. 18 of these genes are predicted
78 to synthesize 13 tRNA modifications in *Mtb* (Supplementary Table 1), including *miaA* and *miaB* for 2-
79 methylthio-6-isopentenyl-adenosine (ms^2i^6A), *tsaD*, *tsaB*, *tsaE*, and *sua5* for N^6-
80 threonylcarbamoyladenosine (t^6A), *mnmA* for 2-thiouridine (s^2U), *truB*, *truA*, and *pus9* for
81 pseudouridine (Ψ), *trmD* for 1-methylguanosine (m^1G), *trmI* for 1-methyladenosine (m^1A), *trmL* for
82 2'-*O*-methylcytidine (Cm) or 2'-*O*-methyluridine (Um), *trmB* for 7-methylguanosine (m^7G), *trmH* for
83 2'-*O*-methylguanosine (Gm), *dusB* for dihydrouridine (D), *tadA* for inosine (I), and *tilS* for lysidine
84 (k^2C). While two additional genes, Rv1713 and Rv2338c, met the threshold for homology to
85 modification enzymes, they exhibited greater similarity to a ribosome associated GTPase (Der) and a
86 molybdopterin biosynthesis protein (MoeW) respectively, and likely do not correspond to tRNA
87 modification enzymes.

88 We mined data from genome-wide Tn-seq and CRISPRi screens[25, 26] to assess the impacts
89 of *Mtb* tRNA modifications on its growth. Five genes encoding *Mtb* tRNA modifying enzymes, *trmD*,
90 *tilS*, *tadA*, *sua5,* and *miaA*, were reported to be essential for *Mtb* growth in both Tn-seq[25] and
91 CRISPRi screens[26] (Supplementary Table 1), suggesting that the modifications they produce are
92 critical for tRNA functions. Indeed, *E. coli trmD, tilS, tadA, and sua5* are also essential for growth and
93 critical for codon decoding, aminoacylation, and reading frame maintenance[27-30]. The *Mtb*
94 modifications synthesized by these enzymes likely have similar impacts on tRNA functions.
95 Unexpectedly, one modifying enzyme, MiaA, is non-essential in *E. coli*, but apparently essential in *Mtb*,

96 suggesting that i^6A, the modification introduced by MiaA, may have more profound roles in *Mtb*
97 translation than in *E. coli*.

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

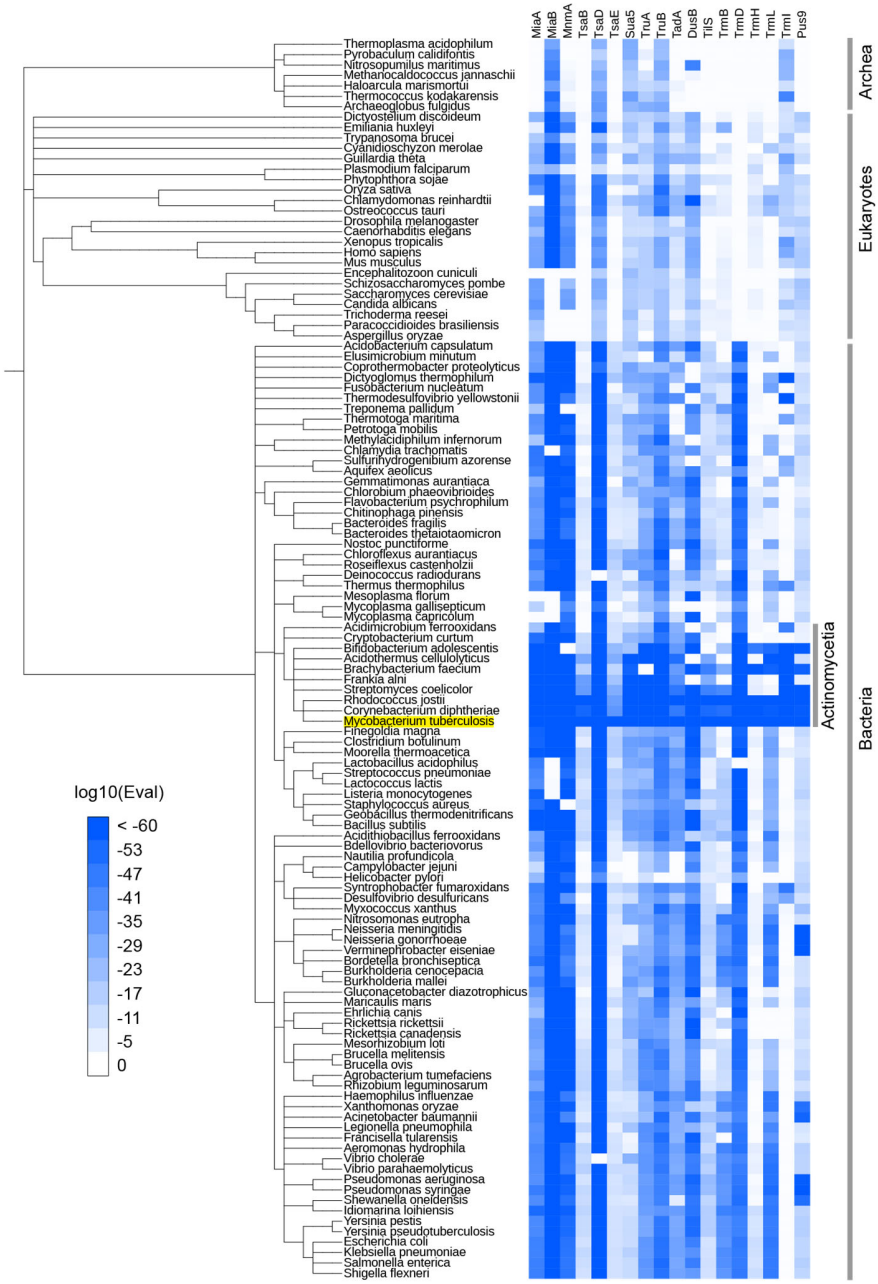

Fig. 1. Phylogenetic distribution of *Mtb* tRNA modifying enzyme homologs. Heat map of log10 E-values from BLAST search results. BLAST searches were conducted against 118 manually picked organisms using *Mtb* tRNA modifying enzymes as queries. When one organism has multiple hits, the lowest log10(Eval) values among hits is shown. iTol[34] was used to depict the results.

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

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

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

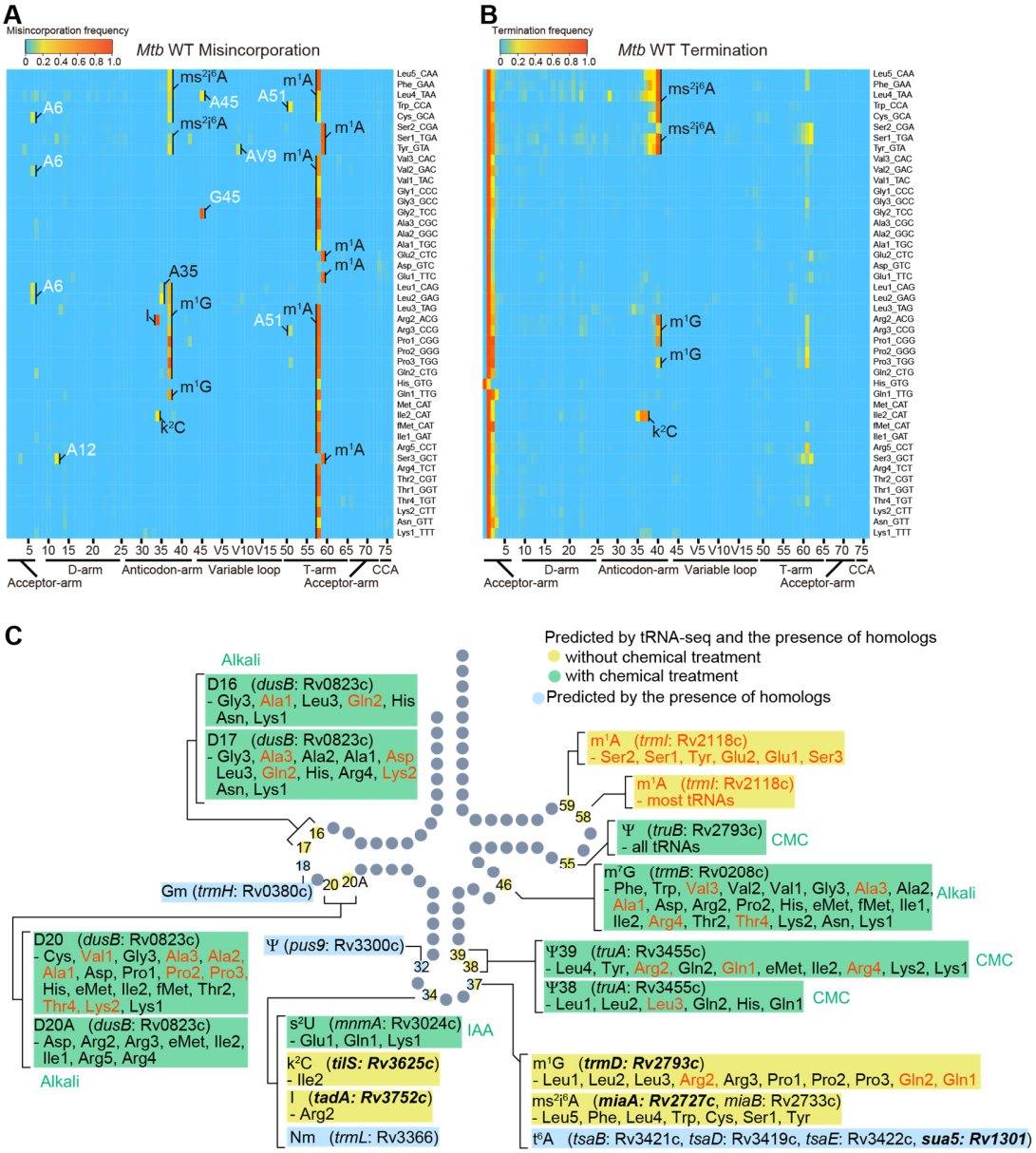

Fig. 2. Heat map of misincorporation and early termination frequency in sequencing of tRNAs from wild type *Mtb*. (A, B) Heatmaps show misincorporation (A) and termination (B) frequencies at all positions across tRNAs (read 5' to 3'). Predicted modifications are labeled based on similarity to known modifications in other organisms and the presence of the tRNA modifying enzyme homologs (Supplementary Table 1). 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.

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

158 IAA is a thiol-reactive compound that covalently attaches carboxyamidomethyl to thiolated
159 uridines via nucleophilic substitution[38] and modified s^4U is detected as C instead of U. In IAA-
160 treated samples, position 8 and 9, corresponding to s^4U in many tRNA species, had high
161 misincorporation frequencies, confirming that IAA treatment modifies s^4U, leading to elevated
162 misincorporation (Supplementary Fig. 1). Furthermore, we observed higher misincorporation and
163 termination signals at the positions corresponding to other sulfur modification, s^2C, s^2U and their
164 derivatives, such as position 32 in tRNA-Arg3, -Arg5, -Ser3, and Arg4, and 34 in tRNA-Glu, -Gln1,
165 and -Lys (Fig. 3 and Supplementary Fig. 1), revealing that IAA treatment facilitates the detection of
166 not only s^4U but also additional sulfur modifications, which are weakly detected without the IAA
167 treatment.

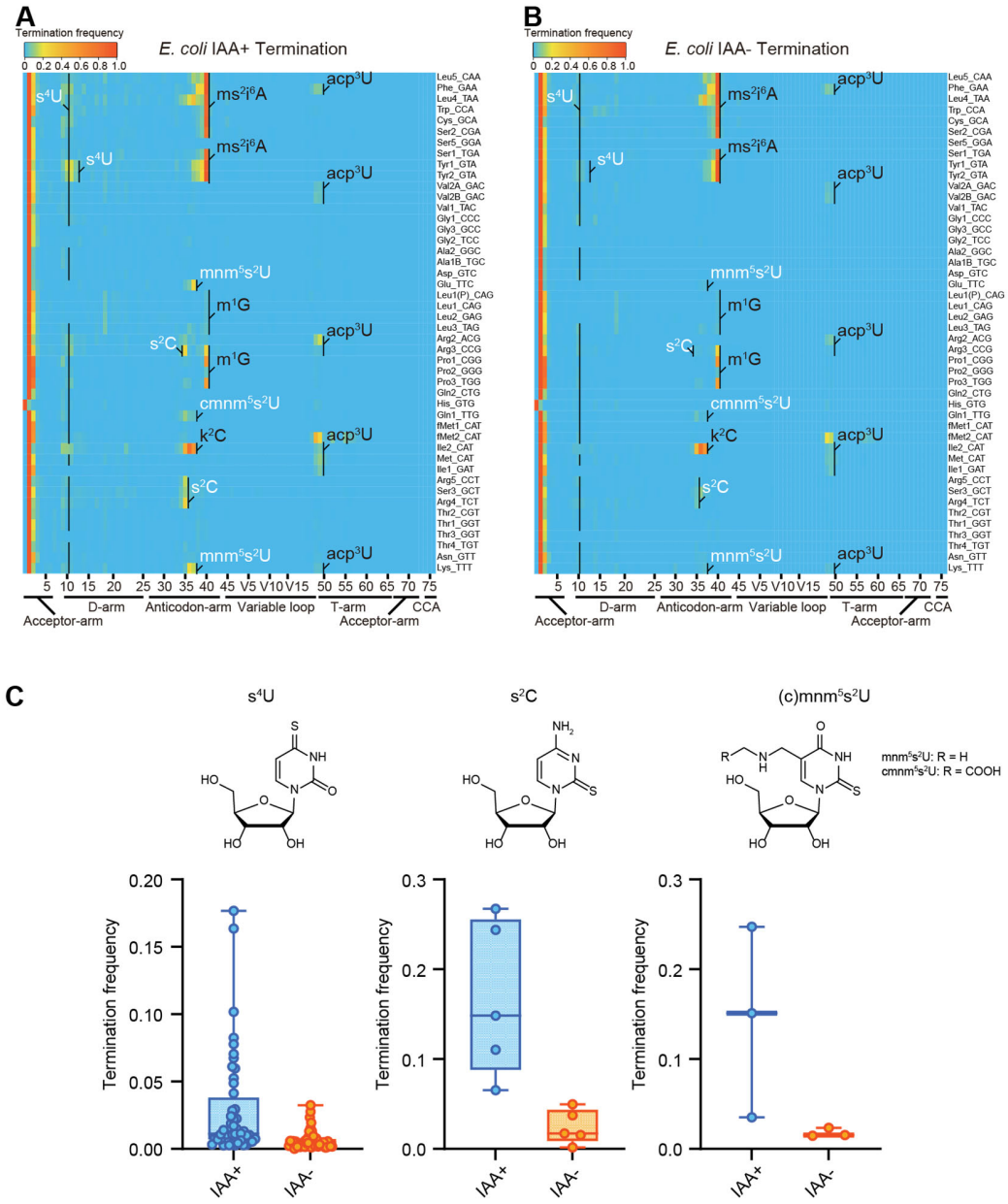

Figure 3. IAA treatment promotes detection of sulfur modifications on tRNAs by enhancing termination signals. (A, B) Heatmaps of the termination signals of *E. coli* tRNAs treated with (A) or without (B) IAA. Known modification sites, including sulfur modifications (s^4U, s^2C, s^2U in white) are shown.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

177 of this enzyme was identified in the *Mtb* genome (Supplementary Table 1)[40]. We used double
178 stranded DNA-based recombineering to delete Rv3024c in *Mtb,* yielding strain *Mtb⊿mnmA*[41].
179 Sequencing of tRNA isolated from *Mtb⊿mnmA* with prior IAA treatment showed reduced termination
180 signals from position 34 in tRNA-Glu1, -Gln1, and -Lys in treated samples, indicating that Rv3024c
181 plays a critical role in the modification responsible for increased termination frequency derived from
182 position 34 in these tRNAs (Fig. 4). Together, these observations strongly suggest that Rv3024c
183 encodes an MnmA-like enzyme that sulfurates position 34 uridines in three *Mtb* tRNA isoacceptors.
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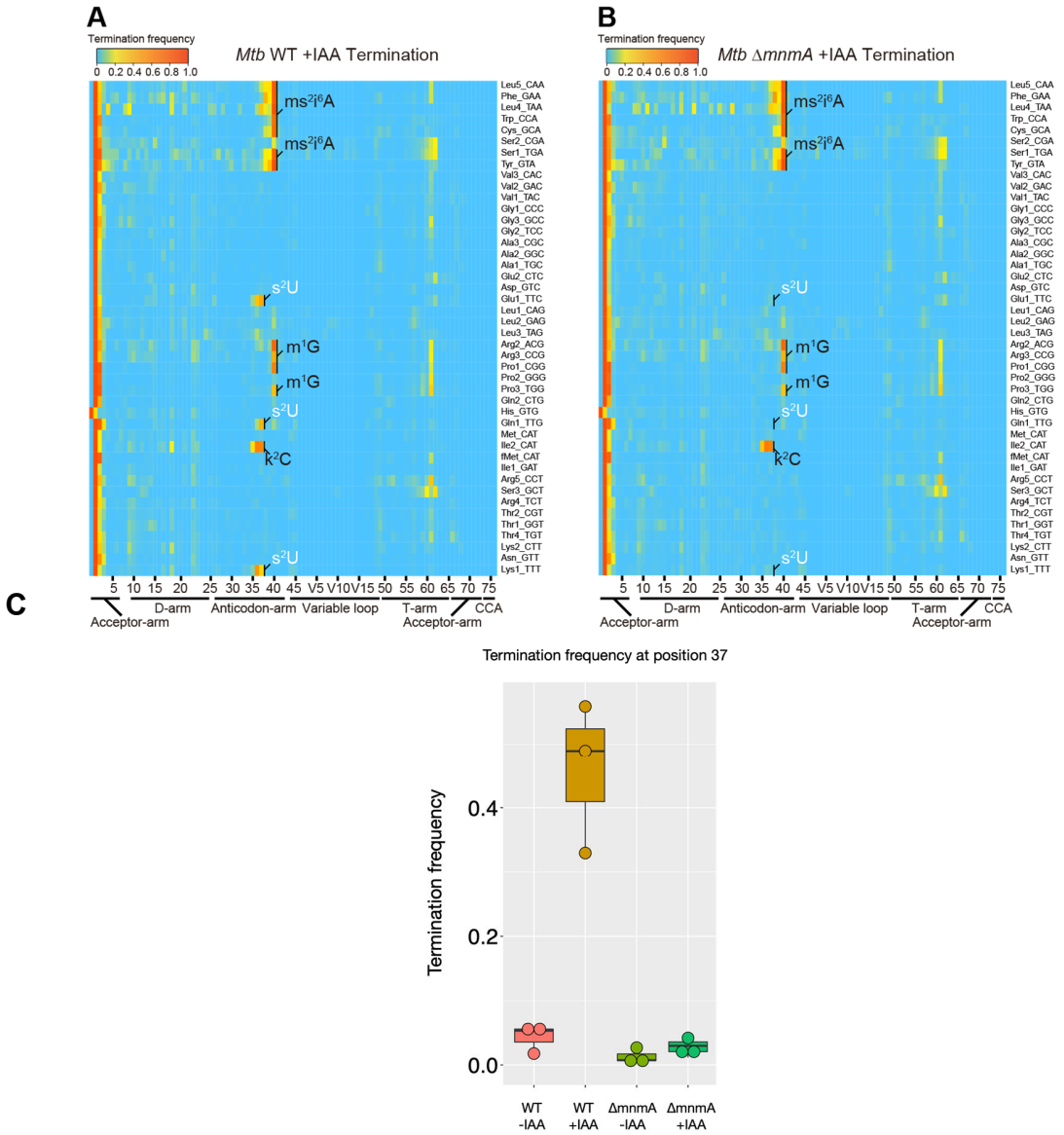

Fig. 4 Heat map and plot of early termination frequency from sequencing of tRNAs from wild type and *MtbΔmnmA* with and without RNA alkylation. (A, B) Heat map of early termination frequencies across tRNA molecules and positions for WT (A) and *Mtb⊿mnmA* (B). Sulfur modification are shown in white. (B)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

As shown previously[42], CMC treatment increased both misincorporation and termination signatures at a subset of Ψs in *E. coli* tRNAs (Fig 5, and Supplementary Fig. 2 and 3). In addition, CMC-treated samples showed increased frequencies of both misincorporation and termination at

197 position 16, 17, 20, and 20A corresponding to D, and m^7G at position 46. Both modifications are
198 known to undergo base elimination in mild alkali conditions[43]. Since these signals were also
199 observed in the reaction condition in which CMC was not added, these signals are likely attributable
200 to the alkali treatment that is common to both conditions.

201 Reverse transcription-derived signatures derived from alkali-treated D showed a distinctive
202 pattern. With this treatment, when two Ds are at consecutive positions, e.g., D16 and D17, termination
203 signals were elevated at the following position (Supplementary Fig. 2 and 4). Furthermore, alkali
204 treatment also led to higher misincorporation frequencies at singlet Ds (Fig. 5 and Supplementary Fig.
205 4). Thus, termination and misincorporation signatures enabled the prediction of known *E. coli* tRNA
206 sites modified to D.

207

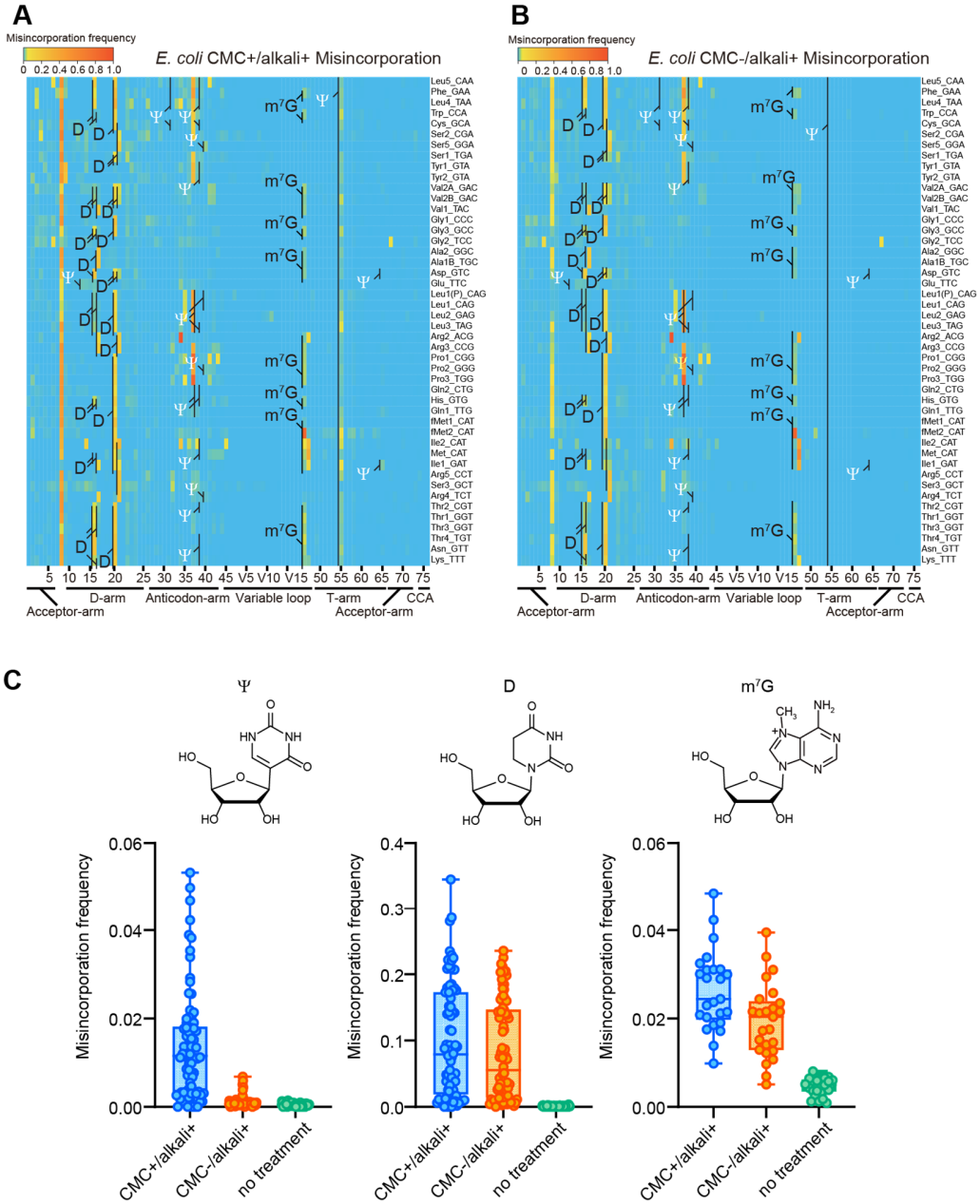

Fig. 5. CMC and alkali treatment facilitate detection of Ψ, D, and m^{7}G modifications in *E. coli*. (A, B) Heatmaps of the misincorporation signals of *E. coli* tRNAs treated with (A) or without (B) CMC. In both conditions, tRNAs are incubated in alkali condition. Known Ψ, D, and m^{7}G sites are shown.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

217
218 CMC/alkali treatment facilitated the identification of additional modifications in *Mtb* tRNAs.
219 Regardless of CMC treatment, alkali treated samples showed increased misincorporation and
220 termination frequencies derived from U located at position 16, 17, 20, and 20A (Fig. 6 and
221 Supplementary Fig. 5). As observed in *E. coli*, termination signals at position 18 and 21 likely
222 correspond to consecutive Ds at position 16 and 17, and 20 and 20A, respectively. *Mtb* Rv0823c is a
223 homolog of dihydrouridylase DusB (Supplementary Table 1), which likely accounts for the synthesis
224 of D at these positions. Furthermore, alkali treatment increased the misincorporation frequencies at
225 G at position 46 (Supplementary Fig. 6). Since Rv0208c is a homolog of TrmB, which synthesizes
226 m^7G at position 46 in *E. coli*, multiple *Mtb* tRNA species likely contain m^7G at position 46 (Fig. 6 and
227 Supplementary Fig. 6).
228 CMC treatment also increased the termination frequency at several sites. Termination
229 signatures derived from position 55 increased in most tRNA species, suggesting that *Mtb* tRNAs
230 contain pseudouridines at this position[37]. Rv2793c is an *Mtb* homologue of *E. coli* TruB and deletion
231 of Rv2793c reduced the termination frequencies at this position in tRNAs isolated from *Mtb*⊿*truB*
232 (Fig. 6 and Supplementary Fig. 7)[41]. Together, these observations suggest that Rv2793c encodes a
233 TruB-like enzyme that modifies position 55 uridines to Ψ across tRNA species. Furthermore, the
234 presence of a TruA homolog in *Mtb* (Rv3455c) suggests that in multiple tRNA species U at positions
235 38-40 can be modified to Ψ. Indeed, the termination signatures derived from positions 38 and 39
236 increased depending on CMC treatment, strongly suggesting that these positions are modified to Ψ
237 (Fig. 6 and Supplementary Fig. 7).
238 In total, among 13 tRNA *Mtb* tRNA modifications predicted by the presence of tRNA
239 modifying enzymes (Fig 1 and Supplementary Table 1), 9 species of modifications were detected based
240 on reverse-transcription derived signatures (Fig. 2C).

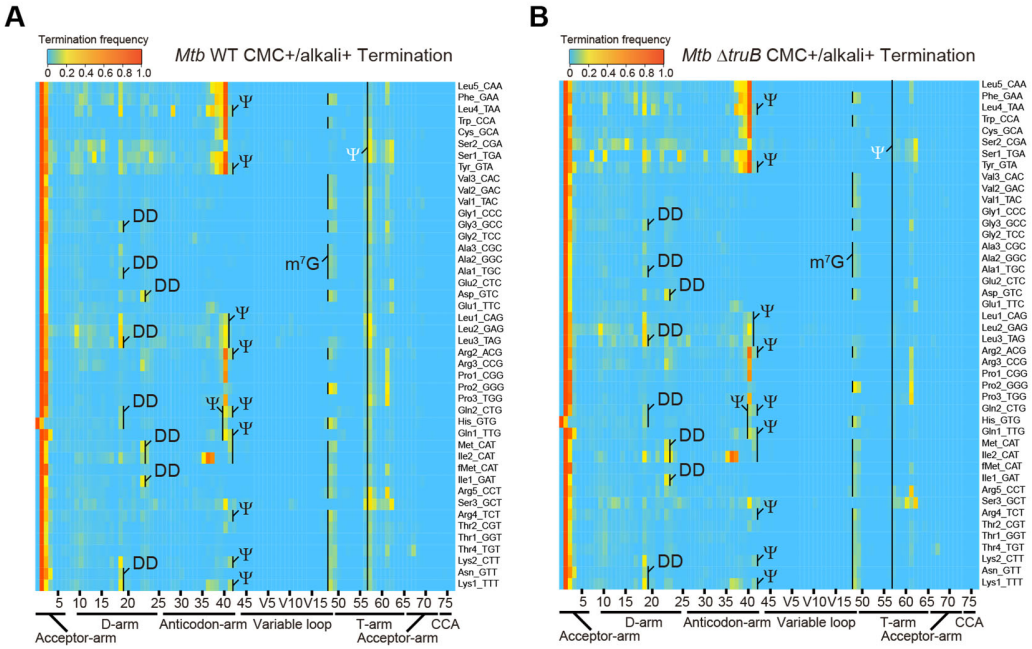

Fig 6. Heat map of early termination frequency from sequencing of tRNAs isolated from WT and *MtbΔtruB* following CMC treatment. Heat map of early termination frequencies across tRNA molecules and positions for WT (left) and *Mtb⊥truB* (right). Termination signals derived from position 55 are shown in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

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

259 macrophages.

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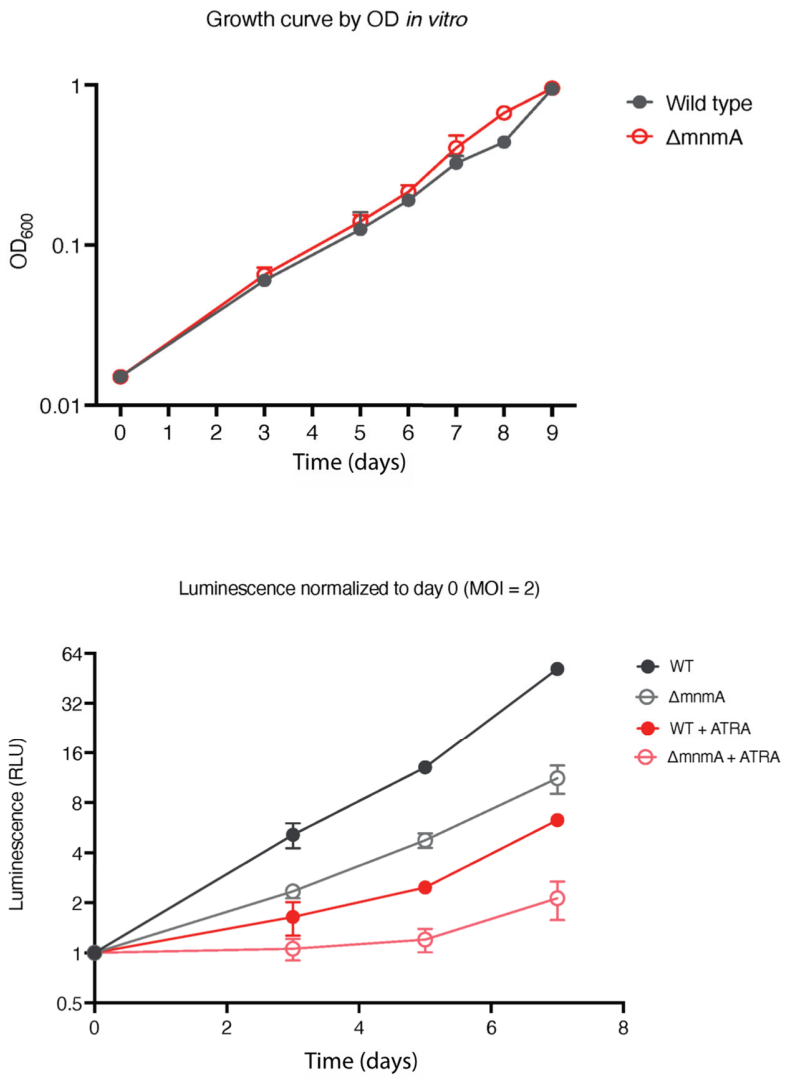

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262 **Fig. 7. *MtbΔmnmA* is attenuated in a macrophage infection model**. Top: Wild type and
263 *MtbΔmnmA* do not display growth differences in 7H9 medium. Bottom: Auto-luminescent wild type
264 and *ΔmnmA Mtb* strains were diluted to a multiplicity of infection of 2 bacteria per mouse bone
265 marrow-derived macrophage with or without all-trans retinoic acid (ATRA). Survival was measured
266 by luminescence and normalized to luminescence at time 0.
267

268

269 **Discussion**

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

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

286 Multiple modifications do not cause reverse transcriptase errors. Although tRNA-seq provides
287 a simple and rapid method for profiling the landscape of modifications in all tRNA species, this
288 approach does not enable comprehensive identification of modifications. Here, several modifications,
289 including t^6A, Cm, Um, and Gm, predicted by the presence of *Mtb* homolog of tRNA modifying
290 enzymes, such as Sua5, TsaB, TsaD, TsaE, TrmL, and TrmH, were not detected in tRNA-seq.
291 Mapping and further analysis of these modifications will require different approaches such as tRNA
292 purification and RNA mass spectrometric analysis.

293 Several strong signatures were detected in *Mtb* tRNAs but not in *E. coli* (Fig. 2A), including
294 G45 in tRNA-Gly2. Most of these signals are strict misincorporations of C or T at A or G position,
295 respectively. Since modifications at a purine position, e.g., m^1G and m^1A, generally cause random
296 misincorporation of the other three nucleosides, strong *Mtb*-specific misincorporation signatures are
297 not likely to be derived from modification. Further mass spectrometric analysis of purified tRNAs will
298 be necessary to assess whether these signals are derived from *Mtb*-specific modifications.

299 The role of s^2U in *Mtb* appears to be unusual. s^2U is a universally conserved modification
300 observed in all three domains of life at the wobble position in the anticodon. This modification
301 enhances the stacking of s^2U with U35 to stabilize the anticodon structure, facilitating codon-
302 anticodon interactions. s^2U is also recognized by multiple aminoacyl-tRNA synthetases for efficient
303 amino acylation[12]. Although elimination of this sulfur modification causes severe growth
304 phenotypes in most organisms[39, 49], unexpectedly, the deletion of *mnmA* in *Mtb* did not attenuate
305 growth of the pathogen *in vitro*, suggesting that the *Mtb* requirement for s^2U modification differs from
306 other organisms. The marked growth retardation of Δ*mnmA* strain within macrophages indicates the
307 specific requirement of this modification within host cells. This modification may be necessary for
308 maintaining general translation efficiency inside host cells and/or facilitate the expression of specific
309 genes that are necessary for survival within macrophages.

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

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

317 offers an efficient and scalable platform for surveying changes in tRNA modifications, this approach
318 will be valuable across growth conditions, and may be extended to growth inside host cells. Finally,
319 further studies elucidating the mechanisms by which tRNA modifications facilitate *Mtb* growth in host
320 cells should be valuable for designing new therapeutics for tuberculosis.
321
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327
328

329 **Materials and Methods**

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

335

336 **Bacterial strain construction:** Supplementary Table 2 depicts the strains, plasmids, primers, and
337 recombinant DNA used for this study. Plasmids were built by restriction digest of a parental vector
338 and inserts were prepared by Gibson assembly[52]. Plasmids were isolated from *E. coli* and confirmed
339 via Sanger sequencing carried out by Genewiz, LLC (Massachusetts, USA).

340
341 Deletion mutants: The knockout strain *Mtb*Δ*mnmA*::zeo (zeocin) was built using double-stranded
342 recombineering in the parental *Mtb* strain H37Rv. A linear dsDNA fragment was constructed using
343 stitch PCR with the primers listed in Supplementary Table 2 which consisted of a 500bp region
344 upstream of *mnmA* (Rv3024c)), 500 bp downstream region, and a *lox*-zeo-*lox* fragment. This cassette
345 was transformed into an H37Rv recombineering strain as described[53] and plated on 7H10 + zeocin
346 plates.

347

348 **Homology search**

349 Local BLAST was performed to search for *Mtb* homologs of tRNA modifying enzymes. First, the
350 uniport IDs of tRNA modifying enzymes were obtained from Modomics[24], and seven proteins were
351 manually added to the list, including Q47319/TapT, P24188/TrhO, P76403/TrhP, O32034/TrhP1,
352 O32035/TrhP2, P36566/CmoM, and Q87K36/TrcP. Uniprot ID provides a fasta file of tRNA

353 modifying enzymes from the Uniprot database[54]. A blast database file was generated by
354 'makeblastdb' script using a fasta file of *Mtb* proteins (H37Rv strain) retrieved from NCBI. Then, the
355 homologs of tRNA modifying enzymes were searched against the *Mtb* protein database using the fasta
356 file of tRNA modifying enzymes as a query. Output format is defined by the following script: -outfmt
357 "7 qacc sacc stitle score qcovs evalue pident" -evalue 1e-10. The output file was modified by excel
358 (Supplementary Table 1).
359

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

361 Local BLAST was conducted to search for homologs of *Mtb* tRNA modifying enzymes in 118
362 manually picked organisms across three domains of life. A custom database was generated by
363 combining the fasta files of organisms' proteins retrieved from NCBI. Homologs of eighteen *Mtb*
364 tRNA modifying enzymes were searched against the custom protein database using local blast.
365 Log10Evalues were visualized by iTol[34] with a phylogenetic tree generated by phyloT[55].
366

367 **tRNA sequencing**

368 Extraction of total RNA: Strains were grown to mid-log phase with the appropriate antibiotics and
369 inducing agents described above. RNA was collected at the same OD_{600} for each strain (between 0.4-
370 0.6). Cells were left on ice for 20 minutes, then pelleted by centrifuging at 4,000 rpm for 10 minutes
371 at 4 °C. Pellets were resuspended in 0.5-1 mL of TriZol (Life Technologies) and lysed using a BeadBug
372 microtube homogenizer (Millipore Sigma). 200 μL of chloroform was added to each tube, after which
373 samples obtained from *Mtb* strains were removed from biosafety level 3 precautions. Samples were
374 centrifuged at 15,000 rpm for 15 minutes at 4 °C and the aqueous layer was collected into a fresh tube.
375 To the original tube, 250 μL of sodium acetate buffer (300 mM sodium acetate pH 5.2 and 10 mM
376 EDTA pH 8.0) was added, and samples were vortexed at 4 °C for 5 minutes then centrifuged at 15,000

377 g for 15 minutes at 4 °C. The aqueous layer was added to the fresh sample-containing tubes. 400 μL
378 chloroform was added, and tubes were briefly vortexed and then centrifuged at 15,000 rpm for 1
379 minute at 4 °C. The aqueous phase was collected into a fresh tube and RNA recovered by ethanol
380 precipitation. RNA pellets were resuspended in 10 mM sodium acetate pH 5.2 and stored at -80 °C
381 until processed for sequencing. Total RNA samples were alkali-treated prior to tRNA extraction to
382 deacylate all tRNAs (1 hour at 37 °C in 100 mM Tris-HCl pH 9.0).
383
384 Isolation of tRNA fraction: 1–2 μg of total RNA was run on a 10% TBE-UREA gel (ThermoFisher
385 Scientific) at 250 V for 1 hour. Gels were stained with SYBR Gold (ThermoFisher Scientific), and
386 tRNA was excised. Excised gels containing tRNA fractions were mashed in RNAse-free tubes, and
387 300 μL elution buffer (300 mM NaOAc pH 5.5, 1 mM EDTA pH 8.0, 0.10% SDS) was added to each
388 tube. Samples were shaken on a thermoshaker (Eppendorf) for 1–4 hours at 37 °C and supernatant
389 was collected using an Ultrafree filter column (Millipore Sigma). tRNA was recovered by isopropanol
390 precipitation.
391
392 tRNA dephosphorylation: tRNA was dephosphorylated using QuickCIP (New England BioLabs)
393 according to manufacturer instructions, and tRNA was collected by phenol-chloroform extraction
394 followed by isopropanol precipitation.
395
396 Iodoacetamide (IAA) treatment: IAA treatment was performed as described[38]. Briefly, 500 ng of
397 total RNA is combined with 10 mM of iodoacetamide, 50 mM $NaPO_4$ pH 8.0, and 50% DMSO in a
398 final volume of 50 μL. Reactions were incubated at 50 °C for 15 minutes and quenched with DTT.
399

400 CMC treatment: CMC treatment was carried out as described in ref. Briefly, 2.5 μg tRNA fraction in
401 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,
402 4 mM EDTA, and 50 mM bicine pH 7.9) and incubated at 37 °C for 20 min. Adding 100 μl CMC
403 stop solution (0.3 M NaOAc pH 5.2 and 100 mM EDTA) quenched the reaction. RNA was desalted
404 with PD-10 desalting column (Cytiva) and recovered by ethanol precipitation. RNA was dissolved in
405 40 μl of 50 mM sodium carbonate buffer (pH 10.4) and incubated at 37 °C for 4 hours, followed by
406 ethanol precipitation.
407
408 Adapter ligation: 0.5 μL RNase inhibitor was added to 3.5μL dephosphorylated tRNA (200–250 ng
409 tRNA) and samples were boiled at 80 °C for 2 minutes. Boiled tRNA was mixed with 12 μL PEG
410 buffer mix (10 μL 50% PEG8000, 2 μL 10 × buffer B0216S; New England Biolabs). 3 μL of 5'
411 adenylated linkers (Supplementary Table 2) were added (33 pmol/μL) along with 1 μL T4 RNA ligase
412 2 truncated (New England BioLabs) and incubated at 25 °C for 2.5 hours. Samples were recovered by
413 isopropanol precipitation and run on a 10% TBE-Urea PAGE gel for 40 minutes at 250 V. Ligated
414 products were recovered by gel excision as described above.
415
416 Reverse transcription: Identical quantities of samples with different adapter sequences were pooled
417 for reverse transcription for a total of 200–250 ng tRNA. Reverse transcription was performed by
418 combining 2.1 μL dephosphorylated tRNA with 100 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1.25 μM
419 RT primer (Supplementary Table 2), 450 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 500 nM TGIRT
420 (InGex), and 15% PEG8000 in a final volume of 9 μL. Samples were incubated at 25 °C for 30
421 minutes, after which 1 μL 10 mM dNTPs (New England BioLabs) were added and reactions incubated
422 at 60 °C for 1 hour. 1.15 μL NaOH was added, and samples were boiled for 15 minutes and run on a

423 10% TBE Urea PAGE gel at 250V for 1 hour. Reverse transcription products were excised from gels
424 and cDNA recovered by isopropanol precipitation. Linear single-stranded cDNA was circularized
425 using CircLigase II (Lucigen) in accordance with manufacturer instructions.
426
427 PCR of tRNA libraries: PCR reactions were set up using HF Phusion according to the manufacturer's
428 instructions using a universal reverse primer (Supplementary Table 2) and a different index primer for
429 each pool of samples. PCR reactions were aliquoted into 4 tubes and collected after 6, 8, 10, and 12
430 cycles. Samples were run on a Native TBE PAGE gel (ThermoFisher Scientific) at 180 V for 50
431 minutes, and amplified products were cut from the same cycle for each sequencing run. Samples were
432 recovered by gel excision and isopropanol precipitation.
433
434 Sequencing: Sequencing was performed on a MiSeq instrument (Illumina) using 150 bp single end
435 reads with a version 3, 150 cycle kit.
436
437 Analysis: 3' linker sequences and two nucleotides at the 5' end were trimmed using cutadapt and fastx-
438 trimmer. Bowtie v1.2.2 was used with default settings to map reads to reference *Mtb* tRNA sequences
439 retrieved from Mycobrowser[40] (Supplementary Table 3). Mpileup files were generated using
440 samtools (samtools mpileup -I -A --ff 4 -x -B -q 0 -d 10000000). For analysis of termination
441 frequencies, 5' end termini of mapped reads were piled up using bedtools genomecov (option, -d -5 -
442 ibam). The number of 5' termini at each tRNA position was divided by the total number of mapped
443 termini at that position plus all upstream (5') positions.
444
445 Macrophage infection: Auto-luminescent wild type and ⊿*mnmA Mtb* strains grown to the same OD_{600}
446 were pelleted by centrifugation and prepared in RPMI media by soft spinning as described[56]. Briefly,

447 cells were washed, pelleted, resuspended, and centrifuged at 121 g, with the top half of the centrifuged
448 supernatant used. Suspensions were diluted to a multiplicity of infection of 2 bacteria per mouse bone
449 marrow-derived macrophage by determining the OD_{600}. Macrophages were infected for 6 hours,
450 followed by a PBS wash and addition of RPMI with or without all-trans retinoic acid (ATRA). ATRA
451 promotes macrophage control of *Mtb* infection[46] and was used to assess strain survival in an
452 increasingly restricted macrophage environment. Survival was measured by luminescence in a BioTek
453 plate reader and normalized to luminescence reads at time 0.
454

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