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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.

eLife assessment

This is a **valuable** addition to the literature as it helps us understand the role of tRNA modifying enzymes in Mycobacterium tuberculosis. By knocking out one of the enzymes, the authors **convincingly** demonstrate the importance of tRNA-modifying enzymes for intra-host growth of tubercle bacteria. Some of the claims regarding modification as well as the role in virulence could be strengthened through further bioinformatics and phylogenetic analyses as well as experimental approaches. The work will be of interest to microbiologists.

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

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

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

Results

In silico prediction of *Mtb* tRNA modifying enzymes

To predict tRNA modifications in *Mtb*, we used Basic Local Alignment Search Tool (BLAST) (23) to identify homologues of all tRNA modification enzymes registered in Modomics in the *Mtb* genome(24). With a stringent threshold (E-value <1×10^{-10}), 20 *Mtb* genes homologous to genes encoding known RNA modification enzymes were identified. 18 of these genes are predicted to synthesize 13 tRNA modifications in *Mtb* (Supplementary Table 1), including *miaA* and *miaB* for 2-methylthio-6-isopentenyl-adenosine (ms^2i^6A), *tsaD, tsaB, tsaE*, and *sua5* for N^6 threonylcarbamoyladenosine (t^6A), *mnmA* for 2-thiouridine (s^2U), *truB, truA*, and *pus9* for pseudouridine (Ψ), *trmD* for 1-methylguanosine (m^1G), *trmI* for 1-methyladenosine (m^1A), *trmL* for 2'-*O*-methylcytidine (Cm) or 2'-*O*-methyluridine (Um), *trmB* for 7-methylguanosine (m^7G), *trmH* for 2'-*O*-methylguanosine (Gm), *dusB* for dihydrouridine (D), *tadA* for inosine (I), and *tilS* for lysidine (k^2C). While two additional genes, Rv1713 and Rv2338c, met the threshold for homology to modification enzymes, they exhibited greater similarity to a ribosome associated GTPase (Der) and a molybdopterin biosynthesis protein (MoeW) respectively, and likely do not correspond to tRNA modification enzymes.

We mined data from genome-wide Tn-seq and CRISPRi screens(25; 26) to assess the impacts of *Mtb* tRNA modifications on its growth. Five genes encoding *Mtb* tRNA modifying enzymes, *trmD, tilS, tadA, sua5*, and *miaA*, were reported to be essential for *Mtb* growth in both Tn-seq(25) and CRISPRi screens(26) (Supplementary Table 1), suggesting that the modifications they produce are critical for tRNA functions. Indeed, *E. coli trmD, tilS, tadA, and sua5* are also essential for growth and critical for codon decoding, aminoacylation, and reading frame maintenance[(27)-(30)]. The *Mtb* modifications synthesized by these enzymes likely have similar impacts on tRNA functions. Unexpectedly, one modifying enzyme, MiaA, is non-essential in *E. coli*, but apparently essential in *Mtb*, suggesting that i^6A, the modification introduced by MiaA, may have more profound roles in *Mtb* translation than in *E. coli*.

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

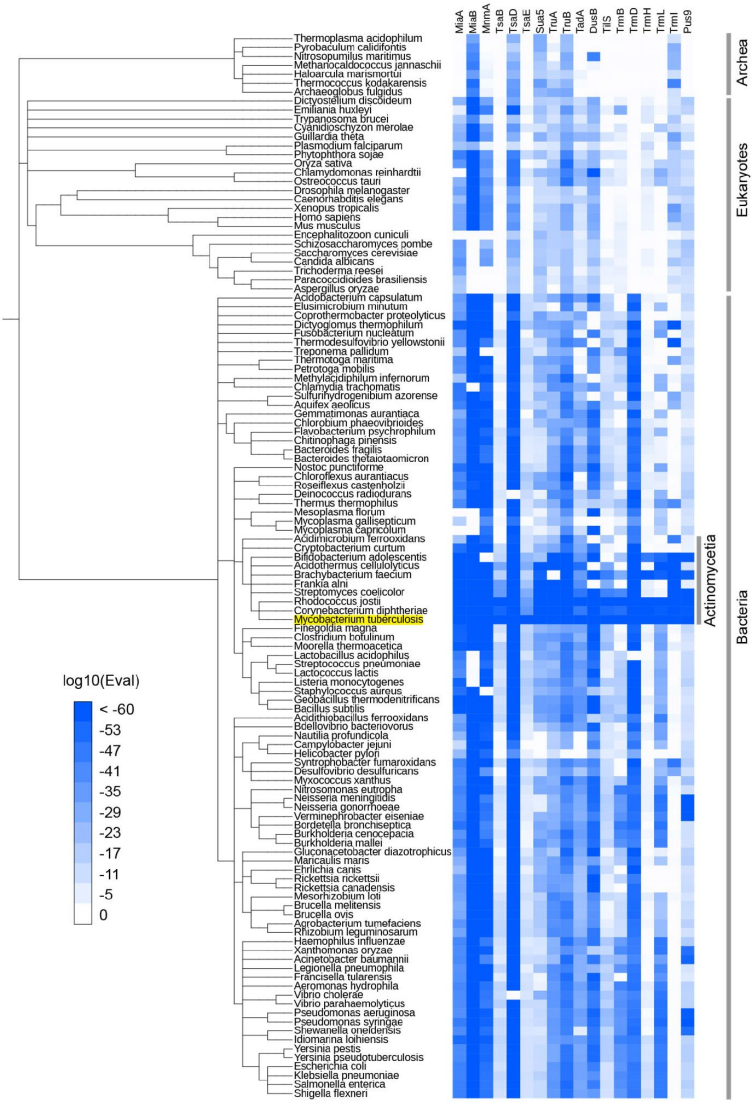

Fig. 1.

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.

Profiling *Mtb* tRNA modification sites by tRNA sequencing

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

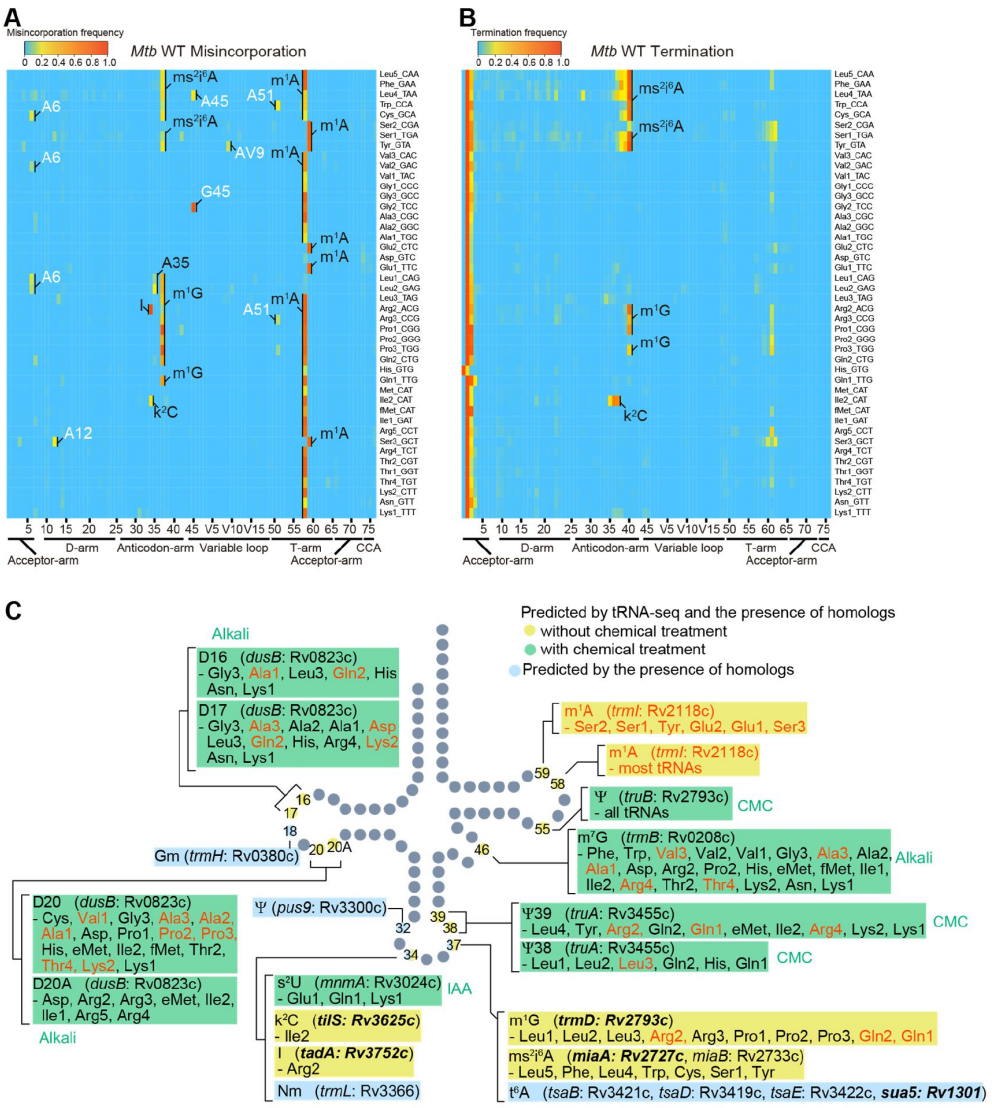

Fig. 2.

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.

Comparison of the reverse transcription derived signatures observed in *Mtb* to *E. coli*, in which tRNA modifications are previously well characterized(16) enables the prediction of the presence of common modifications, including ms^2i^6A, m^1G, I, and k^2C (Fig 2). These predictions are strongly supported by the set of tRNA modification enzymes identified in the *Mtb* genome (Fig. 1 and Supplementary Table 1), including *miaA* and *miaB* (ms^2i^6A), *trmD* (m^1G), *tadA* (I), and *tilS* (k^2C). Some tRNA modifications were observed in *Mtb* but are not present in *E. coli*. In other actinobacteria, A58 and A59 are likely modified to m^1A(36), and since *trmI*, the methylase that generates this modification is present in *Mtb*(33), most *Mtb* tRNAs likely contain this modification as well. Nucleoside variation in the sequence of tRNA genes can account for some of the variations in modified sites between *E. coli* and *Mtb*. For example, in *Mtb*, termination signatures derived from G at position 37 were detected in

during reverse transcription, these positions are likely modified to m^1G, as observed in the *Bacillus subtilis* tRNA-Arg2 gene position 37 G(37).

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

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

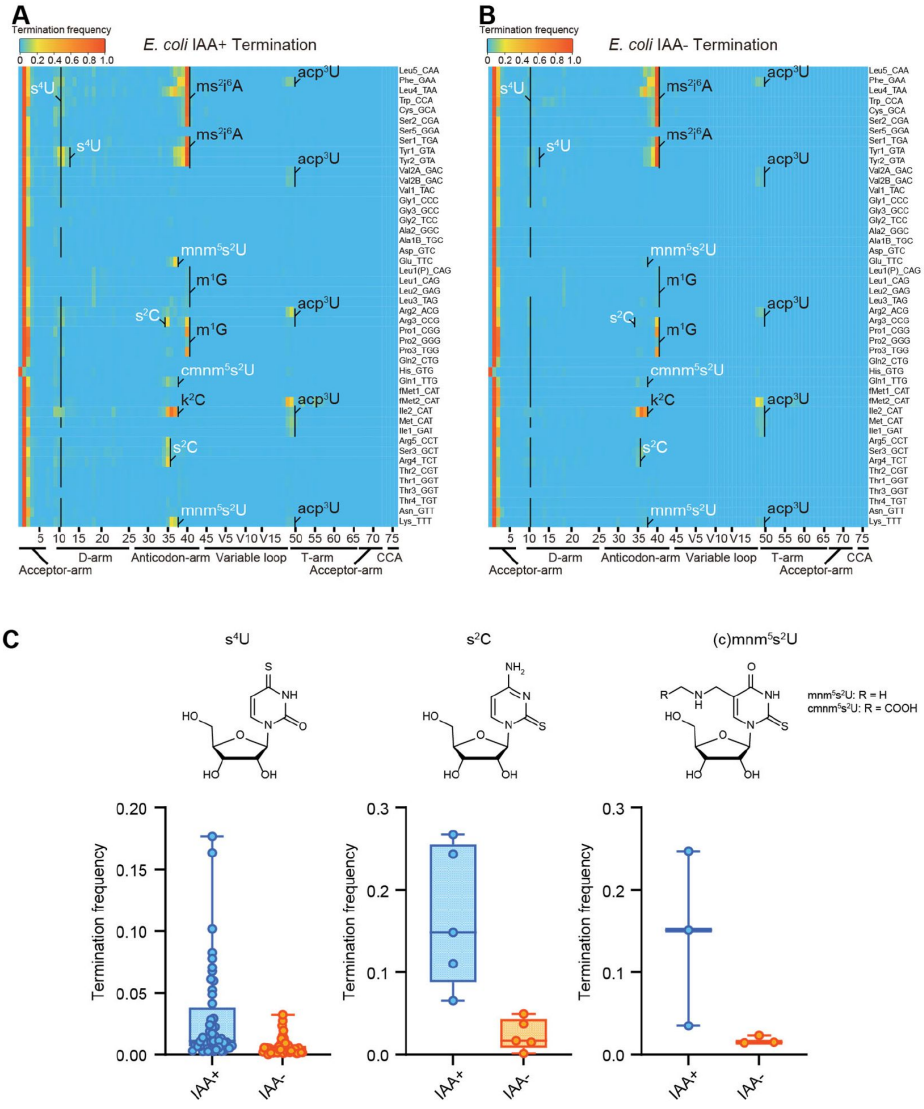

Figure 3.

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

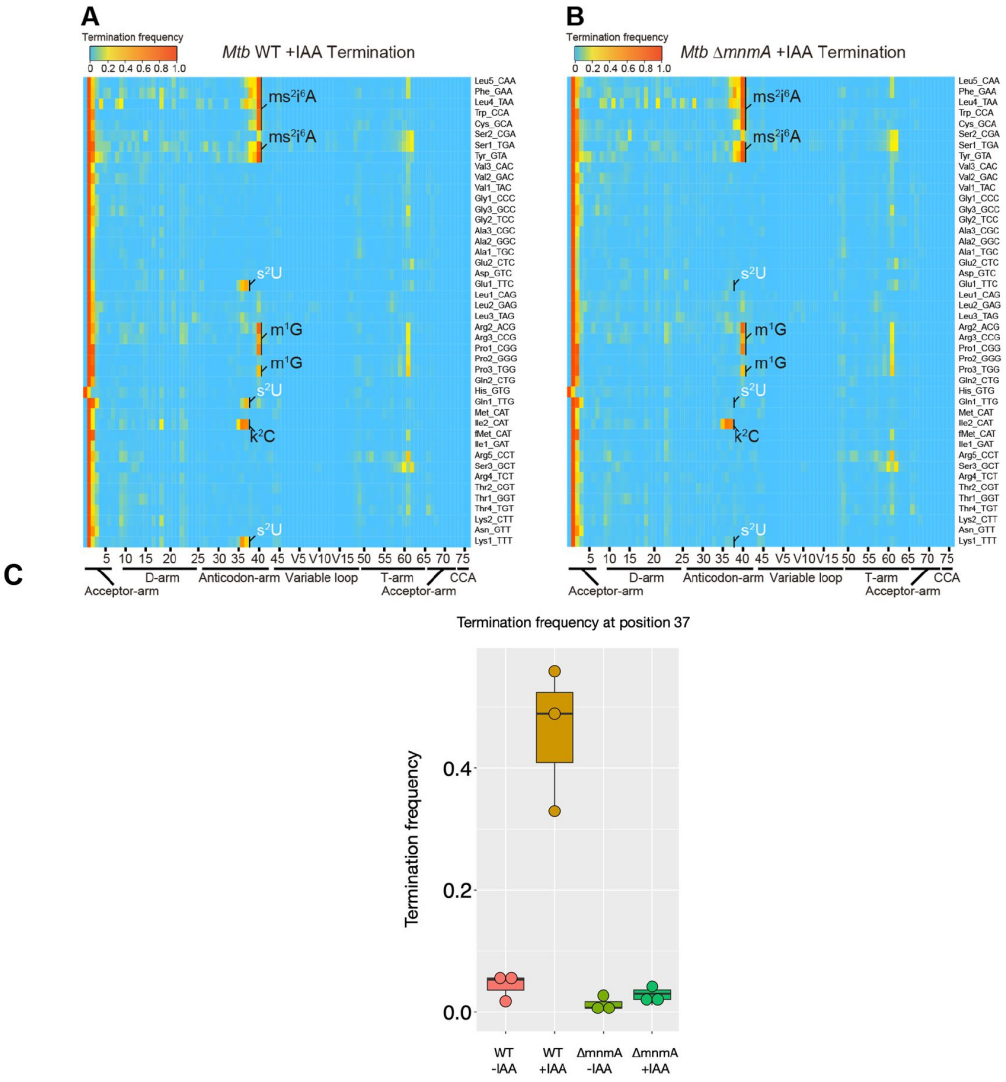

Fig. 4.

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 *MtbΔmnmA* (B). Sulfur modification are shown in white. (B) Plot of termination frequencies at position 37 in wild type (WT) *Mtb* and *MtbΔmnmA* 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 position 16, 17, 20, and 20A corresponding to D, and m^7G at position 46. Both modifications are known to undergo base elimination in mild alkali conditions(43). Since these signals were also observed in the reaction condition in which CMC was not added, these signals are likely attributable to the alkali treatment that is common to both conditions.

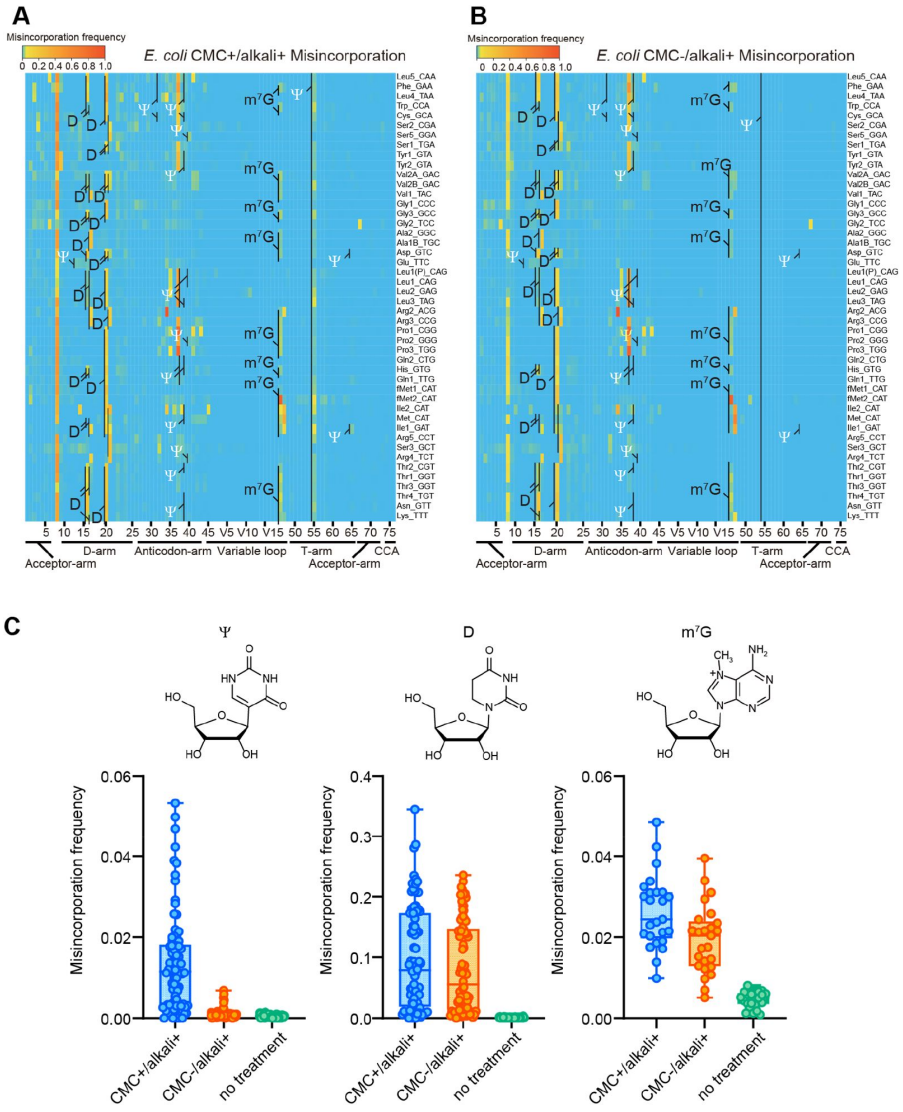

Fig. 5.

CMC and alkali treatment facilitate detection of Ψ, D, and m^7G 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^7G sites are shown. Ψ are shown in white. (C) Misincorporation frequency at known Ψ, D, m^7G sites of tRNAs treated with CMC+/alkali+ or CMC-/alkali+, and tRNAs without treatment.

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

CMC/alkali treatment facilitated the identification of additional modifications in *Mtb* tRNAs. Regardless of CMC treatment, alkali treated samples showed increased misincorporation and termination frequencies derived from U located at position 16, 17, 20, and 20A (Fig. 6 and Supplementary Fig. 5). As observed in *E. coli*, termination signals at position 18 and 21 likely correspond to consecutive Ds at position 16 and 17, and 20 and 20A, respectively. *Mtb* Rv0823c is a homolog of dihydrouridylase DusB (Supplementary Table 1), which likely accounts for the synthesis of D at these positions. Furthermore, alkali treatment increased the misincorporation frequencies at G at position 46 (Supplementary Fig. 6). Since Rv0208c is a homolog of TrmB, which synthesizes m^7G at position 46 in *E. coli*, multiple *Mtb* tRNA species likely contain m^7G at position 46 (Fig. 6 and Supplementary Fig. 6).

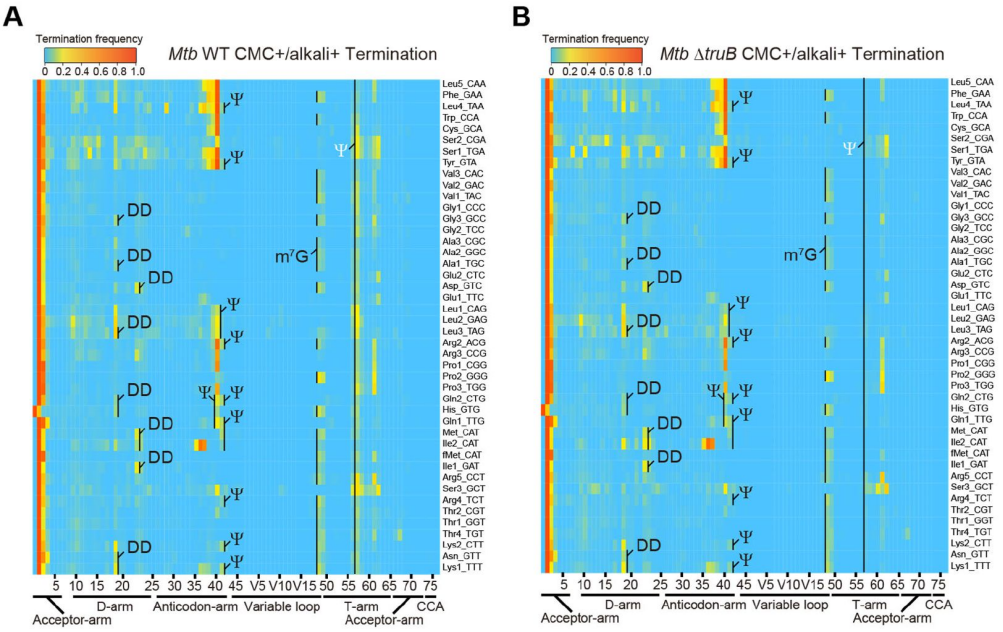

Fig 6.

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 *MtbΔtruB* (right). Termination signals derived from position 55 are shown in white.

CMC treatment also increased the termination frequency at several sites. Termination signatures derived from position 55 increased in most tRNA species, suggesting that *Mtb* tRNAs contain pseudouridines at this position(37). Rv2793c is an *Mtb* homologue of *E. coli* TruB and deletion of Rv2793c reduced the termination frequencies at this position in tRNAs isolated from *MtbΔtruB* (Fig. 6 and Supplementary Fig. 7)(41). Together, these observations suggest that Rv2793c encodes a TruB-like enzyme that modifies position 55 uridines to Ψ across tRNA species. Furthermore, the presence of a TruA homolog in *Mtb* (Rv3455c) suggests that in multiple tRNA species U at positions 38-40 can be modified to Ψ. Indeed, the termination signatures derived from positions 38 and 39 increased depending on CMC treatment, strongly suggesting that these positions are modified to Ψ (Fig. 6 and Supplementary Fig. 7).

In total, among 13 tRNA *Mtb* tRNA modifications predicted by the presence of tRNA modifying enzymes (Fig 1 and Supplementary Table 1), 9 species of modifications were detected based on reverse-transcription derived signatures (Fig. 2C).

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 *MtbΔmnmA* 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 *MtbΔmnmA* mutant was significantly impaired for growth in a macrophage infection model(45) (Fig. 7). Defective growth of the *MtbΔmnmA* mutant was also observed in macrophages treated with all-trans retinoic acid (ATRA), which promotes macrophage control of *Mtb* infection(46). These

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

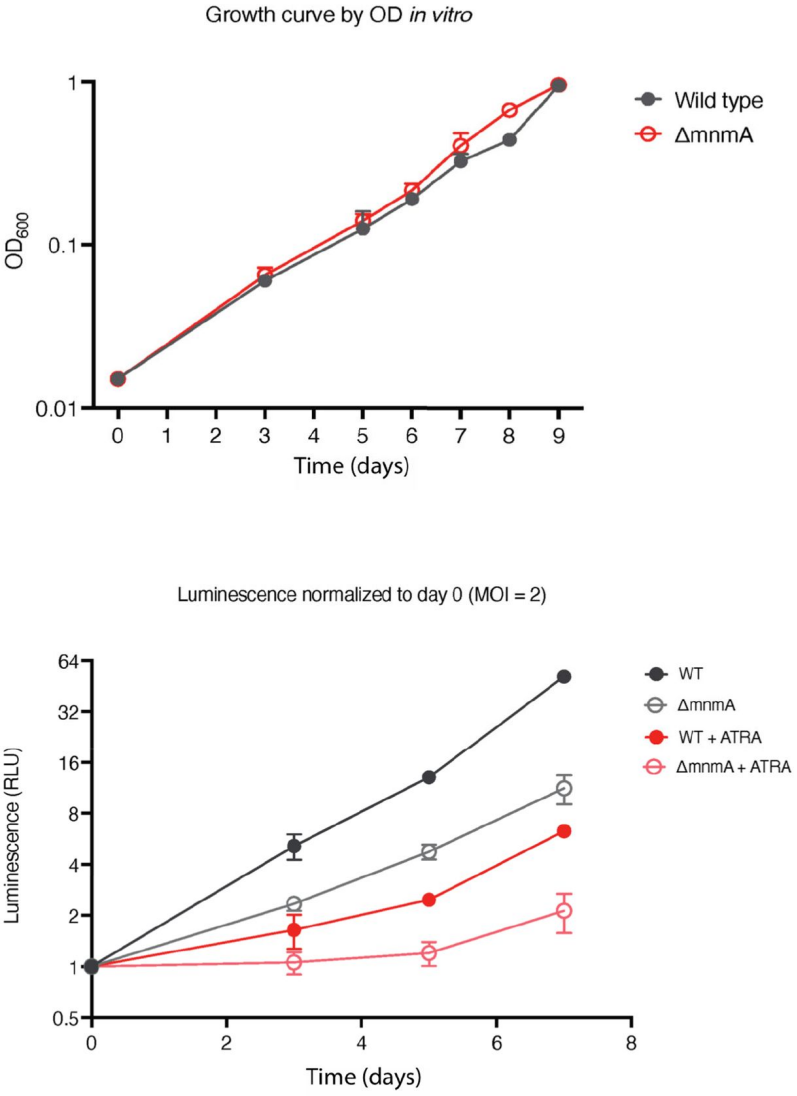

Fig. 7.

MtbΔmnmA is attenuated in a macrophage infection model.

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

Discussion

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

additional sulfur modifications as well, including s^2C and s^2U, indicating that IAA should have general utility in tRNA-seq-based profiling of sulfur modifications, including in studies tracking changes in tRNA sulfuration(47; 48).

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

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

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

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

Our findings serve as a valuable starting point for the research community to continue characterizing the physiological roles and mechanisms of *Mtb* tRNA modifications. Since tRNA-seq offers an efficient and scalable platform for surveying changes in tRNA modifications, this approach will be valuable across growth conditions, and may be extended to growth inside host cells. Finally, further studies elucidating the mechanisms by which tRNA modifications facilitate *Mtb* growth in host cells should be valuable for designing new therapeutics for tuberculosis.

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Materials and Methods

Bacterial strains and growth conditions

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

Bacterial strain construction

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

Deletion mutants

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

Homology search

Local BLAST was performed to search for *Mtb* homologs of tRNA modifying enzymes. First, the uniport IDs of tRNA modifying enzymes were obtained from Modomics(24), and seven proteins were manually added to the list, including Q47319/TapT, P24188/TrhO, P76403/TrhP, O32034/TrhP1, O32035/TrhP2, P36566/CmoM, and Q87K36/TrcP. Uniprot ID provides a fasta file of tRNA modifying enzymes from the Uniprot database(54). A blast database file was generated by 'makeblastdb' script using a fasta file of *Mtb* proteins (H37Rv strain) retrieved from NCBI. Then, the homologs of tRNA modifying enzymes were searched against the *Mtb* protein database using the fasta file of tRNA modifying enzymes as a query. Output format is defined by the following script: -outfmt "7 qacc sacc stitle score qcovs evalue pident" -evalue 1e-10. The output file was modified by excel (Supplementary Table 1).

Phylogenetic analysis of *Mtb* tRNA modifying enzyme homologs

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

tRNA sequencing

Extraction of total RNA

Strains were grown to mid-log phase with the appropriate antibiotics and inducing agents described above. RNA was collected at the same OD_{600} for each strain (between 0.4-0.6). Cells were left on ice for 20 minutes, then pelleted by centrifuging at 4,000 rpm for 10 minutes at 4 °C. Pellets were resuspended in 0.5-1 mL of TriZol (Life Technologies) and lysed using a BeadBug microtube homogenizer (Millipore Sigma). 200 µL of chloroform was added to each tube, after which samples obtained from *Mtb* strains were removed from biosafety level 3 precautions. Samples were centrifuged at 15,000 rpm for 15 minutes at 4 °C and the aqueous layer was collected into a fresh tube. To the original tube, 250 µL of sodium acetate buffer (300 mM sodium acetate pH 5.2 and 10 mM EDTA pH 8.0) was added, and samples were vortexed at 4 °C for 5 minutes then centrifuged at 15,000 *g* for 15 minutes at 4 °C. The aqueous layer was added to the fresh sample-containing tubes. 400 µL chloroform was added, and tubes were briefly vortexed and then centrifuged at 15,000 rpm for 1 minute at 4 °C. The aqueous phase was collected into a fresh tube and RNA recovered by ethanol precipitation. RNA pellets were resuspended in 10 mM sodium acetate pH 5.2 and stored at -80 °C until processed for sequencing. Total RNA samples were alkali-treated prior to tRNA extraction to deacylate all tRNAs (1 hour at 37 °C in 100 mM Tris-HCl pH 9.0).

Isolation of tRNA fraction

1–2 µg of total RNA was run on a 10% TBE-UREA gel (ThermoFisher Scientific) at 250 V for 1 hour. Gels were stained with SYBR Gold (ThermoFisher Scientific), and tRNA was excised. Excised gels containing tRNA fractions were mashed in RNAse-free tubes, and 300 µL elution buffer (300 mM NaOAc pH 5.5, 1 mM EDTA pH 8.0, 0.10% SDS) was added to each tube. Samples were shaken on a thermoshaker (Eppendorf) for 1–4 hours at 37 °C and supernatant was collected using an Ultrafree filter column (Millipore Sigma). tRNA was recovered by isopropanol precipitation.

tRNA dephosphorylation

tRNA was dephosphorylated using QuickCIP (New England BioLabs) according to manufacturer instructions, and tRNA was collected by phenol-chloroform extraction followed by isopropanol precipitation.

Iodoacetamide (IAA) treatment

IAA treatment was performed as described(38). Briefly, 500 ng of total RNA is combined with 10 mM of iodoacetamide, 50 mM $NaPO_4$ pH 8.0, and 50% DMSO in a final volume of 50 µL. Reactions were incubated at 50 °C for 15 minutes and quenched with DTT.

CMC treatment

CMC treatment was carried out as described in ref. Briefly, 2.5 µg tRNA fraction in 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, 4 mM EDTA, and 50 mM bicine pH 7.9) and incubated at 37 °C for 20 min. Adding 100 µl CMC stop solution (0.3 M NaOAc pH 5.2 and 100 mM EDTA) quenched the reaction. RNA was desalted with PD-10 desalting column (Cytiva) and recovered by ethanol precipitation. RNA was dissolved in 40 µl of 50 mM sodium carbonate buffer (pH 10.4) and incubated at 37 °C for 4 hours, followed by ethanol precipitation.

Adapter ligation

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

Reverse transcription

Identical quantities of samples with different adapter sequences were pooled for reverse transcription for a total of 200–250 ng tRNA. Reverse transcription was performed by combining 2.1 µL dephosphorylated tRNA with 100 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1.25 µM RT primer (Supplementary Table 2), 450 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 500 nM TGIRT (InGex), and 15% PEG8000 in a final volume of 9 µL. Samples were incubated at 25 °C for 30 minutes, after which 1 µL 10 mM dNTPs (New England BioLabs) were added and reactions incubated at 60 °C for 1 hour. 1.15 µL NaOH was added, and samples were boiled for 15 minutes and run on a 10% TBE Urea PAGE gel at 250V for 1 hour. Reverse transcription products were excised from gels and cDNA recovered by isopropanol precipitation. Linear single-stranded cDNA was circularized using CircLigase II (Lucigen) in accordance with manufacturer instructions.

PCR of tRNA libraries

PCR reactions were set up using HF Phusion according to the manufacturer's instructions using a universal reverse primer (Supplementary Table 2) and a different index primer for each pool of samples. PCR reactions were aliquoted into 4 tubes and collected after 6, 8, 10, and 12 cycles. Samples were run on a Native TBE PAGE gel (ThermoFisher Scientific) at 180 V for 50 minutes, and amplified products were cut from the same cycle for each sequencing run. Samples were recovered by gel excision and isopropanol precipitation.

Sequencing

Sequencing was performed on a MiSeq instrument (Illumina) using 150 bp single end reads with a version 3, 150 cycle kit.

Analysis

3' linker sequences and two nucleotides at the 5' end were trimmed using cutadapt and fastx-trimmer. Bowtie v1.2.2 was used with default settings to map reads to reference *Mtb* tRNA sequences retrieved from Mycobrowser(40) (Supplementary Table 3). Mpileup files were generated using samtools (samtools mpileup -I -A --ff 4 -x -B -q 0 -d 10000000). For analysis of termination frequencies, 5' end termini of mapped reads were piled up using bedtools genomecov (option, -d -5 - ibam). The number of 5' termini at each tRNA position was divided by the total number of mapped termini at that position plus all upstream (5') positions.

Macrophage infection

Auto-luminescent wild type and *ΔmnmA Mtb* strains grown to the same OD_{600} were pelleted by centrifugation and prepared in RPMI media by soft spinning as described(56). Briefly,

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

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Reviewer #1 (Public Review):

Tomasi et al. performed a combination of bioinformatic, next-generation tRNA sequencing experiments to predict the set of tRNA modifications and their corresponding genes in the tRNAs of the pathogenic bacteria Mycobacterium tuberculosis. Long known to be important for translation accuracy and efficiency, tRNA modifications are now emerging as having regulatory roles. However, the basic knowledge of the position and nature of the modifications present in a given organism is very sparse beyond a handful of model organisms. Studies that can generate the tRNA modification maps in different organisms along the tree of life are good starting points for further studies. The focus here on a major human pathogen that is studied by a large community raises the general interest of the study. Finally, deletion of the gene mnmA responsible for the insertion of s2U at position 34 revealed defects in in growth in macrophage but in test tubes suggesting regulatory roles that will warrant further studies. The conclusions of the paper are mostly supported by the data but the partial nature of the bioinformatic analysis and absence of Mass-Spectrometry data make it incomplete. The authors do not take advantage of the Mass spec data that is published for Mycobacterium bovis (PMID: 27834374) to discuss what they find.

Important points to be considered:

1. The authors say they took a list of proteins involved in tRNA modifications from Modomics and added manually a few but we do not know the exact set of proteins that were used to search the M. mycobacterium genome.

2. The absence of mnmGE genes in TB suggested that the xcm5U derivatives are absent. These are present in M. bovis (PMID: 27834374). Are the MnmEG gene found in M. bovis? If yes, then the authors should perform a phylogenetic distribution analysis in the Mycobacterial clade to see when they disappeared. If they are not present in M.

bovis then maybe a non-orthologous set of enzymes do the same reaction and then the authors really do not know what modification is present or not at U34 without LC-MS. The exact same argument can be given for the xmo5U derivatives that are also found in M.bovis but not predicted by the authors in M. tuberculosis.

3. Why is the Psi32 predicted by the authors because of the presence of the Rv3300c/Psu9 gene not detected by CMC-treated tRNA seq while the other Psi residues are? Members of this family can modify both rRNA and tRNA. So the presence of the gene does not guarantee the presence of the modification in tRNAs

4. What are tsaBED not essential but tsaC (called sua5 by the authors) essential?

Reviewer #2 (Public Review):

In this study, Tomasi et al identify a series of tRNA modifying enzymes from Mtb, show their function in the relevant tRNA modifications and by using at least one deleted strain for MnmA, they show the relevance of tRNA modification in intra-host survival and postulate their potential role in pathogenesis.

Conceptually it is a wonderful study, given that tRNA modifications are so fundamental to all life forms, showing their role in Mtb growth in the host is significant. However, the authors have not thoroughly analyzed the phenotype. The growth defect aspect or impact on pathogenesis needs to be adequately addressed.

- The authors show that ΔmnmA grows equally well in the in vitro cultures as the WT. However, they show attenuated growth in the macrophages. Is it because Glu1_TTC and Gln1-TTG tRNAs are not the preferred tRNAs for incorporation of Glu and Gln, respectively? And for some reason, they get preferred over the alternate tRNAs during infection? What dictates this selectivity?

- As such the growth defect shown in macrophages would be more convincing if the authors also show the phenotype of complementation with WT mnmA.

An important consideration here is the universal nature of these modifications across the life forms. Any strategy to utilize these enzymes as the potential therapeutic candidate would have to factor in this important aspect.

Reviewer #3 (Public Review):

The work presented in the manuscript tries to identify tRNA modifications present in Mycobacterium tuberculosis (Mtb) using reverse transcription-derived error signatures with tRNA-seq. The study identified enzyme homologs and correlates them with presence of respective tRNA modifications in Mtb. The study used several chemical treatments (IAA and alkali treatment) to further enhance the reverse transcription signals and confirms the presence of modifications in the bases. tRNA modifications by two enzymes TruB and MnmA were established by doing tRNA-seq of respective deletion mutants. Ultimately, authors show that MnmA-dependent tRNA modification is important for intracellular growth of Mtb. Overall, this report identifies multiple tRNA modifications and discuss their implication in Mtb infection.

Important points to be considered:

- The presence of tRNA-based modifications is well characterised across life forms including genus Mycobacterium (Mycobacterium tuberculosis: Varshney et al, NAR, 2004; Mycobacterium bovis: Chionh et al, Nat Commun, 2016; Mycobacterium abscessus: Thomas

et al, NAR, 2020). These modifications are shown to be essential for pathogenesis of multiple organisms. A comparison of tRNA modification and their respective enzymes with host organism as well as other mycobacterium strains is required. This can be discussed in detail to understand the role of common as well as specific tRNA modifications implicated in pathogenesis.

- Authors state in line 293 "Several strong signatures were detected in Mtb tRNAs but not in E. coli". Authors can elaborate more on the unique features identified and their relevance in Mtb infection in the discussion or result section.

- Deletion of MnmA is shown to be essential for E. coli growth under oxidative stress (Zhao et al, NAR, 2021). In similar lines, MnmA deleted Mtb suffers to grow in macrophage. Is oxidative stress in macrophage responsible for slow Mtb growth?

- Authors state in line 311-312 "Mtb does not contain apparent homologs of the tRNA modifying enzymes that introduce the additional modifications to s2U". This can be characterised further to rule out the possibility of other enzyme specifically employed by Mtb to introduce additional modification.

Author Response:

Reviewer #1 (Public Review):

Tomasi et al. performed a combination of bioinformatic, next-generation tRNA sequencing experiments to predict the set of tRNA modifications and their corresponding genes in the tRNAs of the pathogenic bacteria Mycobacterium tuberculosis. Long known to be important for translation accuracy and efficiency, tRNA modifications are now emerging as having regulatory roles. However, the basic knowledge of the position and nature of the modifications present in a given organism is very sparse beyond a handful of model organisms. Studies that can generate the tRNA modification maps in different organisms along the tree of life are good starting points for further studies. The focus here on a major human pathogen that is studied by a large community raises the general interest of the study. Finally, deletion of the gene mnmA responsible for the insertion of s2U at position 34 revealed defects in in growth in macrophage but in test tubes suggesting regulatory roles that will warrant further studies. The conclusions of the paper are mostly supported by the data but the partial nature of the bioinformatic analysis and absence of Mass-Spectrometry data make it incomplete. The authors do not take advantage of the Mass spec data that is published for Mycobacterium bovis (PMID: 27834374) to discuss what they find.

Important points to be considered:

1. *The authors say they took a list of proteins involved in tRNA modifications from Modomics and added manually a few but we do not know the exact set of proteins that were used to search the M. mycobacterium genome.*

Thank you for pointing out this issue. We will add the complete list of proteins used for the BLAST query.

> 2. *The absence of mnmGE genes in TB suggested that the xcm5U derivatives are absent. These are present in M. bovis (PMID: 27834374). Are the MnmEG gene found in M. bovis? If yes, then the authors should perform a phylogenetic distribution analysis in the Mycobacterial clade to see when they disappeared. If they are not present in M. bovis then maybe a non-orthologous set of enzymes do the same reaction and then the authors really do not know what modification is present or not at U34 without LC-MS. The exact same argument can be given for the xmo5U derivatives that are also found in M.bovis but not predicted by the authors in M. tuberculosis.*

The reviewer raises a valid point. In M. bovis mnm5U and cmo5U derivatives were observed in LC-MS analysis. However, we did not identify candidate genes known to be involved in the biogenesis of mnm5U and cmo5U in the Mycobacteriaceae, including M. bovis and Mtb, suggesting that if these modifications are indeed present, they are not synthesized through a canonical biogenesis pathways in this family. There are several examples where the same modification is generated by distinct modification enzymes (Kimura, 2021). These observations raise the interesting possibility that in the Mycobacteriaceae and most species in actinomycetota (except for Bifidobacterium, Corynebacterium and Rhodococcus species), major wobble modifications are generated by biosynthesis pathways that are distinct from those employed by well-characterized organisms. Future studies will examine this hypothesis.

> 3. *Why is the Psi32 predicted by the authors because of the presence of the Rv3300c/Psu9 gene not detected by CMC-treated tRNA seq while the other Psi residues are? Members of this family can modify both rRNA and tRNA. So the presence of the gene does not guarantee the presence of the modification in tRNAs*

Thank you very much for the careful read. We did not include RluA in the list of query proteins because it is not classified as a tRNA modification enzyme in Modomics. Additionally, the CMC-coupled tRNA-seq is imperfect for detection of all pseudouridylated positions. Due to this limitation, we only assigned modifications that are both predicted by the presence of putative biosynthetic enzymes and RT-derived signatures. As the reviewer points out, we cannot rule out that this homolog targets only rRNAs. We will clarify this possibility in the revised manuscript. Also, RluA will be added to the query and the name of Rv3300c will be changed to RluA in the text and related figures.

> 4. *What are tsaBED not essential but tsaC (called sua5 by the authors) essential?*

Thank you for pointing out this interesting observation. We are also curious about differences in the essentiality among t6A biogenesis genes. We speculate that TsaC potentially has critical roles in cell viability other than t6A synthesis. TsaC synthesizes a compound, threonylcarbamoyl-AMP, as an intermediate for t6A biogenesis. Thus, it is possible that this intermediate has a role in other essential cellular activities besides t6A biogenesis. Further study of these factors in Mtb could reveal interesting crosstalk between modification synthesis and other cellular activities.

> ***Reviewer #2 (Public Review):***
>
> *In this study, Tomasi et al identify a series of tRNA modifying enzymes from Mtb, show their function in the relevant tRNA modifications and by using at least one deleted strain for MnmA, they show the relevance of tRNA modification in intra-host survival and postulate their potential role in pathogenesis.*
>
> *Conceptually it is a wonderful study, given that tRNA modifications are so fundamental to all life forms, showing their role in Mtb growth in the host is significant. However, the authors have not thoroughly analyzed the phenotype. The growth defect aspect or impact on pathogenesis needs to be adequately addressed.*

> *- The authors show that ΔmnmA grows equally well in the in vitro cultures as the WT. However, they show attenuated growth in the macrophages. Is it because Glu1_TTC and Gln1-TTG tRNAs are not the preferred tRNAs for incorporation of Glu and Gln, respectively? And for some reason, they get preferred over the alternate tRNAs during infection? What dictates this selectivity?*

Thank you very much for raising this excellent point. As the reviewer suggests, the attenuation of DmnmA Mtb growth inside of macrophages could be caused by disparate codon usage between genes required for in vitro growth and intracellular growth. Among multiple codons encoding Glu, Gln, or Lys, s2U modification-dependent codons might be preferentially distributed in genes associated with intracellular growth. For example, Mtb has two tRNA isoacceptors, Glu1_TTC and Glu2_CTC, to decipher two Glu codons, i.e., GAA and GAG. According to the wobble pairing rule, GAA is only decoded by Glu1_TTC, whereas GAG is decoded by both Glu1_TTC and Glu2_CTC; i.e., GAG can be deciphered by an s2U-independent tRNA. Thus, genes required for intracellular growth might be enriched with GAA, an s2U-dependent codon. The same thing can happen to other Gln and Lys codons deciphered by s2U-containing tRNAs. In the revised manuscript, we will include the perspective of codon usage for explaining the intracellular fitness defect of the ΔmnmA Mtb mutant.

> *- As such the growth defect shown in macrophages would be more convincing if the authors also show the phenotype of complementation with WT mnmA.*

The reviewer raises a valid point. We note however, that Rv3023c, a putative transposase, is downstream of MnmA and unlike MnmA, Rv3023c appears to be dispensable for in vivo growth, according to the Tn-seq database. Therefore, it is likely that the intracellular growth defect is caused by loss of mnmA.

> *An important consideration here is the universal nature of these modifications across the life forms. Any strategy to utilize these enzymes as the potential therapeutic candidate would have to factor in this important aspect.*

This is a valid point. Targeting a pathogen-specific system enables avoidance of the adverse side effects caused by many therapeutic reagents. There are a couple of Mtb modification enzymes that are specific to bacteria and critical for Mtb fitness (e.g., TilS). These enzymes represent ideal potential therapeutic targets to suppress Mtb intracellular growth.

> ***Reviewer #3 (Public Review):***
>
> *The work presented in the manuscript tries to identify tRNA modifications present in Mycobacterium tuberculosis (Mtb) using reverse transcription-derived error signatures with tRNA-seq. The study identified enzyme homologs and correlates them with presence of respective tRNA modifications in Mtb. The study used several chemical treatments (IAA and alkali treatment) to further enhance the reverse transcription signals and confirms the presence of modifications in the bases. tRNA modifications by two enzymes TruB and MnmA were established by doing tRNA-seq of respective deletion mutants. Ultimately, authors show that MnmA-dependent tRNA modification is important for intracellular growth of Mtb. Overall, this report identifies multiple tRNA modifications and discuss their implication in Mtb infection.*
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> *- The presence of tRNA-based modifications is well characterised across life forms including genus Mycobacterium (Mycobacterium tuberculosis: Varshney et al, NAR, 2004; Mycobacterium bovis: Chionh et al, Nat Commun, 2016; Mycobacterium abscessus: Thomas et al, NAR, 2020). These modifications are shown to be essential for pathogenesis of multiple*

> *organisms. A comparison of tRNA modification and their respective enzymes with host organism as well as other mycobacterium strains is required. This can be discussed in detail to understand the role of common as well as specific tRNA modifications implicated in pathogenesis.*

The reviewer raises a fair point. However, with the exception of Chionh et al., the other studies cited here are not genome-wide characterization of tRNA modification. We will add a discussion of the distribution of tRNA modification enzymes across multiple mycobacterium species and the implications of this distribution for pathogenesis to the revised manuscript.

> *- Authors state in line 293 "Several strong signatures were detected in Mtb tRNAs but not in E. coli". Authors can elaborate more on the unique features identified and their relevance in Mtb infection in the discussion or result section.*

Thank you for the suggestion. We will lengthen the discussion of the RT-derived signatures observed in Mtb but not in E. coli but the relevance of these modifications for Mtb pathogenicity remains speculative at this point.

> *- Deletion of MnmA is shown to be essential for E. coli growth under oxidative stress (Zhao et al, NAR, 2021). In similar lines, MnmA deleted Mtb suffers to grow in macrophage. Is oxidative stress in macrophage responsible for slow Mtb growth?*

This is an excellent hypothesis which we will raise in the revised manuscript.

> *- Authors state in line 311-312 "Mtb does not contain apparent homologs of the tRNA modifying enzymes that introduce the additional modifications to s2U". This can be characterised further to rule out the possibility of other enzyme specifically employed by Mtb to introduce additional modification.*

The reviewer raises a valid point. As discussed above (Reviewer #1, pt 2), Mtb may employ distinct enzymes to generate certain tRNA modifications. Future mass spec-based analyses of Mtb tRNAs will be carried out to identify the precise chemical structure of the sulfurated uridine, and subsequent studies will attempt to determine the enzymes that account for the biogenesis of these modifications.