Development of Assay Systems for Amber Codon Decoding at the Steps of Initiation and Elongation in Mycobacteria

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ABSTRACT

Genetic analysis of the mechanism of protein synthesis in Gram-positive bacteria has remained largely unexplored because of the unavailability of appropriate in vivo assay systems. We developed chloramphenicol acetyltransferase (CAT)-based in vivo reporter systems to study translation initiation and elongation in Mycobacterium smegmatis. The CAT reporters utilize specific decoding of amber codons by mutant initiator tRNA (i-tRNA, metU) molecules containing a CUA anticodon (metUCUA). The assay systems allow structure-function analyses of tRNAs without interfering with the cellular protein synthesis and function with or without the expression of heterologous GlnRS from Escherichia coli. We show that despite their naturally occurring slow-growth phenotypes, the step of i-tRNA formylation is vital in translation initiation in mycobacteria and that formylation-deficient i-tRNA mutants (metUCUA/A1, metUCUA/G72, and metUCUA/G72G73) with a Watson-Crick base pair at the 1-72 position participate in elongation. In the absence of heterologous GlnRS expression, the mutant tRNAs are predominantly aminoacylated (glutamylated) by nondiscriminating GluRS. Acid urea gels show complete transamidation of the glutamylated metUCUA/G72G73 tRNA to its glutaminylated form (by GatCAB) in M. smegmatis. In contrast, the glutamylated metUCUA/G72 tRNA did not show a detectable level of transamidation. Interestingly, the metUCUA/A1 mutant showed an intermediate activity of transamidation and accumulated in both glutamylated and glutaminylated forms. These observations suggest important roles for the discriminator base position and/or a weak Watson-Crick base pair for in vivo recognition of the glutamylated tRNAs by M. smegmatis GatCAB.

IMPORTANCE

Genetic analysis of the translational apparatus in Gram-positive bacteria has remained largely unexplored because of the unavailability of appropriate in vivo assay systems. We developed chloramphenicol acetyltransferase (CAT)-based reporters which utilize specific decoding of amber codons by mutant tRNAs at the steps of initiation and/or elongation to allow structure-function analysis of the translational machinery. We show that formylation of the initiator tRNA (i-tRNA) is crucial even for slow-growing bacteria and that i-tRNA mutants with a CUA anticodon are aminoacylated by nondiscriminating GluRS. The discriminator base position, and/or a weak Watson-Crick base pair at the top of the acceptor stem, provides important determinants for transamidation of the i-tRNA-attached Glu to Gln by the mycobacterial GatCAB.

KEYWORDS

GatCAB, mycobacterium, tRNA, translation

The bulk of our understanding of the mechanism of protein synthesis is derived primarily from Escherichia coli, a Gram-negative bacterium for which reporter-based genetic systems have been well established. However, the mechanism of protein synthesis in Gram-positive bacteria remains largely unexplored. Mycobacteria belong to the class of slow-growing Gram-positive bacteria. A large majority of antibiotics, including those clinically relevant for tuberculosis (TB) treatment, target ribosomes/protein synthesis. With the
rise of multiple-drug-resistant (MDR) and extensively drug-resistant (XDR) TB, development of new antibiotics and systems enabling screening of new classes of small molecules are of critical importance. Despite the development of in vitro systems to study mycobacterial translation (1, 2) and elucidation of cryoelectron microscopy-based structures of mycobacterial ribosomes (3), our understanding of the mechanism of translation in these bacteria has been limited due to their genetic intractability.

Gram-positive systems differ significantly from Gram-negative systems with respect to the components of translation machinery. Mycobacteria, like the other Gram-positive bacteria, lack glutaminyl-tRNA synthetase (GlnRS) and asparaginyl-tRNA synthetase (AsnRS) genes (4). The synthesis of Gln-tRNA<sup>Gln</sup> and Asn-tRNA<sup>Asn</sup> follows a two-step indirect pathway, where tRNA<sup>Gln</sup> and tRNA<sup>Asn</sup> are first misacylated by nondiscriminating glutamyl-tRNA synthetase (ND-GluRS) and aspartyl-tRNA synthetase (ND-AspRS), resulting in production of Glu-tRNA<sup>Gln</sup> and Asp-tRNA<sup>Asn</sup>, respectively (5) (see Fig. S1 in the supplemental material). These misacylated tRNA species are then specifically converted into the correctly charged forms (Gln-tRNA<sup>Gln</sup> and Asn-tRNA<sup>Asn</sup>) by a transamidation reaction involving a heterotrimeric complex, GatCAB, which uses either glutamine or asparagine as the ammonia donor. Mutations in <i>gatA</i> result in increased levels of mistranslation in mycobacteria and phenotypic resistance to rifampin (6, 7).

Both <i>Mycobacterium tuberculosis</i> and <i>Mycobacterium smegmatis</i> possess single-copy initiator tRNA (i-tRNA) genes (<i>metU</i>) as opposed to four in <i>E. coli</i>, albeit under a stronger promoter (8, 9). The sequences of i-tRNAs are identical across all mycobacteria and possess a C1-U72 mismatch at the top of the acceptor stem, as opposed to the C1-A72 mismatch in <i>E. coli</i> i-tRNA (Fig. 1a). Interestingly, mycobacterial i-tRNAs share two
features with the eukaryotic i-tRNAs—the absence of the posttranscriptional modification ribothymidine at position 54 and the presence of 1-methyladenosine at position 58 (10). However, the three critical features known to be important for i-tRNA function in E. coli, i.e., the mismatch at position 1-72, the three consecutive G-C base pairs in the anticodon stem, and the Pu-Py base pair at position 11-24 in the D-arm, are conserved in mycobacteria as well. Mycobacteria also possess a reduced number of rRNA operons (one or two) and possess a large fraction of mRNAs, which are translated by leaderless initiation (11).

In E. coli, genetic studies on the structure-function relationship of i-tRNA (tRNA^{Met}) and its derivatives in translation initiation and elongation have been made possible by the availability of in vivo assay systems (12, 13). For example, the CAT_{am1} and CAT_{am27} reporters in E. coli utilize chloramphenicol acetyltransferase (CAT) genes with UAG codons at positions relevant to measure in vivo initiation and elongation activities of tRNAs possessing CUA anticodons. The i-tRNA mutants with a CUA anticodon are recognized by glutaminyl-tRNA synthetase (GlnRS) instead of methionyl-tRNA synthetase (MetRS), for which the CAU anticodon is a major determinant. Initiation using i-tRNA with a CUA anticodon occurs with formylglutamine (12).

In this study, we developed CAT reporter-based systems for M. smegmatis by cloning i-tRNA gene mutants together with CAT_{am1}, CAT_{am9}, and CAT_{am27} genes, obtained insights into the recognition of the tRNAs by ND-GluRS, GctCAB, and formylase, and established the utility of the genetic assay systems to study the translation apparatus in mycobacteria.

RESULTS

In vivo reporter systems for initiation and elongation in mycobacteria. To investigate the structure-function relationship of translation apparatus components in M. smegmatis, we developed plasmid-borne reporter systems comprising CAT genes under the constitutive promoter of M. tuberculosis metU and possessing amber codons at defined locations for decoding by the i-tRNA mutants having a CUA anticodon. The CAT reporter (9) was cloned into an E. coli-mycobacterium shuttle vector, pMV261 (Kan') (14).

Mutants of the E. coli i-tRNA with a CUA anticodon are known to be aminoacylated with glutamine by GlnRS (12). However, mycobacteria lack GlnRS. In vitro aminoacylation reactions with purified E. coli GlnRS (EcoGlnRS) and the in vivo aminoacylation status of metU_{CUA} in the background of EcoGlnRS expression in M. smegmatis mc²^{15}QS (harboring the EcoGlnRS gene on an integrative vector, pMV361, at the L5 att region) indicated that the E. coli enzyme is capable of recognizing and aminoacylating it with glutamine (see Fig. S2 in the supplemental material). Thus, we relied on the expression of heterologous EcoGlnRS to aminoacylate the metU_{CUA} tRNA. Heterologous expression of EcoGlnRS did not confer any toxicity to M. smegmatis; rather, it slightly improved growth compared to the vector control (Fig. 1b).

For the initiation assay, the CAT reporter (CAT_{am1}) is specifically decoded by metU_{CUA} tRNA (Fig. 1c). Transformation of the episomal reporter construct pMV_CAT_{am1} metU_{CUA} into M. smegmatis mc²^{15}QS enabled growth on chloramphenicol-containing plates (up to 90 μg/ml) (Fig. 2a, sectors 7 and 8). The CAT levels in the cell extracts were confirmed by immunoblot analysis using anti-CAT antibodies (Fig. 2b). As expected, an ~25-kDa band corresponding to CAT was detected in mc²^{15}QS/pMV_CAT_{am1} metU_{CUA} but was absent in mc²^{15}QS/pMV_CAT_{am1} extracts. CAT assays using [¹⁴C]chloramphenicol further confirmed the initiation activity of metU_{CUA} tRNA (Fig. 2c).

EcoGlnRS-independent initiation with metU_{CUA}. Interestingly, we observed that initiation of translation in the CAT_{am1} reporter in M. smegmatis could occur independently of EcoGlnRS expression (Fig. 2a, sectors 5 and 6; Fig. 2b, lane 3; and 2c, bar 4). As a control, cells transformed with pMV_CAT_{am1} do not grow on chloramphenicol (Fig. 2a, sectors 3 and 4). These observations show that the translation of the CAT reporter is due exclusively to decoding of the UAG start codon by the metU_{CUA} tRNA and suggest that metU_{CUA} might be recognized by a cellular aminoacyl-tRNA synthetase and subsequently by the formylase. Analysis of the in vivo aminoacylation status of metU_{CUA} by acid urea polyacrylamide gel electrophoresis (PAGE), which separates the
deacylated tRNA from its aminoacylated and formylated forms (15), showed that about 10% of metU_CUA tRNA existed in an aminoacylated state in vivo even in the absence of heterologous EcoGlnRS, compared to about an 80% aminoacylated form with EcoGlnRS expression (Fig. 2d), confirming that the metU_CUA tRNA is indeed a substrate for a cellular aminoacyl-tRNA synthetase. Immunoblot analysis showed expression of CAT protein, as expected (Fig. 2b), and CAT activity assays indicated that the CAT expression in the absence of EcoGlnRS expression was about 4-fold lower than with EcoGlnRS expression (Fig. 2c), indicating that aminoacylation and formylation of the tRNA might be limiting in the absence of EcoGlnRS expression.

metU_CUA tRNA is aminoacylated by MetRS and ND-GluRS. To identify the aminoacyl tRNA synthetase which aminoacylates metU_CUA tRNA, we performed in vitro aminoacylation assays using dialyzed S100 extracts from M. smegmatis in the presence of single amino acids. We also cloned and partially purified 15 of the 18 aminoacyl-tRNA synthetases from M. tuberculosis (Fig. S3) to perform activity assays on the mutant tRNA. However, we could not detect aminoacylation in either case, possibly due to the low efficiency of aminoacylation of the tRNA by the enzymes in vitro.

We then adopted a mass spectrometric approach to directly identify the amino acid inserted into the reporter protein. For this purpose, we constructed a new reporter, pMV_CAT_{am1-His} metU_CUA (Fig. 1d), where the amber codon is followed by a hexahistidine tag, enabling purification of the reporter protein. We purified the reporter protein from M. smegmatis mc²155S and mc²155QS (Fig. S4) and subjected it to in-gel trypsin digestion, followed by enrichment of the N-terminal peptide by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography. Analysis of these fractions by mass spectrometry showed a 1,137.5-Da peak in the reporter protein purified from an EcoGlnRS-expressing strain (mc²155QS) (Fig. 3a). This corresponds to a mass that is 17 Da less than that expected for glutamine insertion at the first position. As the free N termini of proteins containing glutamine are known to become cyclized with the loss of ammonia (17 Da) to form pyroglutamate (16), we could assign the peak to pyroglutamate. The identity

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**FIG 2** Initiation using the CAT_{am1} metU_CUA reporter system. (a) Growth of M. smegmatis mc²155 or M. smegmatis mc²155QS containing the reporter plasmid on kanamycin or kanamycin plus chloramphenicol plates. (b) Immunoblot of cell extracts (30 µg total proteins) from reporter-expressing strains using anti-CAT antibodies. (c) CAT activity assays performed using cell extracts (~50 ng total proteins) from the reporter-expressing strains and ¹⁴C-labeled chloramphenicol. (d) In vivo aminoacylation and formylation status of metU_CUA analyzed with or without overexpression of EcoGlnRS by acid urea PAGE, followed by Northern blot analysis.
of the peak was further confirmed by tandem mass spectrometry (MS/MS) analysis (Fig. S5a). We hypothesize that subsequent to initiation with formylglutamine and removal of the formyl group by the deformylase enzyme, the exposed glutamine at the N-terminal gets cyclized. Interestingly, we also detected minor peaks corresponding to 1,182.5 Da (Fig. 3a; Fig. S5b) for formylglutamine at the first position. This may be the result of incomplete removal of the formyl group from the initiating formylglutamine, which would not cyclize.

In the case of the reporter protein purified from the strain lacking the EcoGlnRS gene (mc2155), we detected two major peaks corresponding to 1,157.5 Da and 1,137.5 Da (Fig. 3b). The 1,157.5-Da peak corresponds to the mass expected if methionine is inserted at the first position in the protein, while the 1,137.5-kDa peak corresponds to pyroglutamate as described above. The identity of both peaks was further confirmed by MS/MS analysis (Fig. S5c). We conclude that metU CUA is aminoacylated by two different aminoacyl-tRNA synthetases, i.e., MetRS and ND-GluRS, in M. smegmatis. The results were further confirmed by in vitro aminoacylation with metU CUA and purified EcoMetRS or M. tuberculosis MetRS (MtuMetRS), where we detect a faint band corresponding to methionine charged i-tRNA CUA (Met-metU CUA) (Fig. S6). However, we were unsuccessful in obtaining in vitro aminoacylation with purified MtuGluRS (Fig. S7), which could be attributed to the low efficiency of in vitro aminoacylation of metU CUA RNA.

Formylation-deficient mutants of the i-tRNA participate at the step of elongation. No native suppressor tRNAs have been described so far in any mycobacteria. In the case of E. coli i-tRNA, mutations in the acceptor stem that result in a Watson-Crick
base pair at position 1-72 make the i-tRNA formylation deficient and enable it to participate at the step of elongation (17). Therefore, to construct a system for suppression analysis, we mutated the acceptor stem of the M. tuberculosis i-tRNA to convert the C1-U72 mismatch into a base pair. Guided by our earlier studies in E. coli (17), we made three mutants: A1 (A1·U72 base pair), G72 (C1·G72 base pair), and G72G73 (C1·G72 base pair, with an additional A-to-G mutation in the discriminator base) (Fig. 4a). Analysis of the in vivo aminoacylation and formylation status revealed that these tRNAs are indeed deficient in formylation (Fig. 4b). We analyzed the initiation efficiency with these acceptor stem mutants using the CAT$_{am1}$ reporter system and quantified by CAT activity assays using cell extracts (50 to 200 ng total proteins) from the reporter-expressing strains and $^{14}$C-labeled chloramphenicol. (d) Elongation efficiencies of the acceptor stem mutants measured by the CAT$_{am27}$ reporter system and quantified by CAT activity assays using cell extracts (≈2 μg total proteins) from the reporter-expressing strains and $^{14}$C-labeled chloramphenicol.

FIG 4 Elongation by formylation-deficient mutants of the initiator tRNA. (a) Acceptor stem mutants of i-tRNA tested for elongation. (b) In vivo aminoacylation and formylation status of the i-tRNA mutants as analyzed by acid urea PAGE, followed by Northern blotting. Bands marked with Q, E, O, and * correspond to their glutaminylated, glutamylated, deacylated, and formylaminocacylated populations, identified by their resistance/sensitivity to mild (CuSO$_4$) or strong (Tris, pH 9) alkali. (c) Initiation efficiencies with the acceptor stem mutants using the CAT$_{am1}$ reporter system and quantified by CAT activity assays using cell extracts (50 to 200 ng total proteins) from the reporter-expressing strains and $^{14}$C-labeled chloramphenicol. (d) Elongation efficiencies of the acceptor stem mutants measured by the CAT$_{am27}$ reporter system and quantified by CAT activity assays using cell extracts (≈2 μg total proteins) from the reporter-expressing strains and $^{14}$C-labeled chloramphenicol.

Recognition of acceptor stem mutants by MtuGluRS. Comparison of the migration patterns of the tRNA mutants revealed that the acceptor stem mutants are aminoacylated better than the metU$_{CUA}$ tRNA. Further, the migration of aminoacylated

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fractions of the tRNAs were different. To investigate the identity of the aminoacyl tRNA synthetase(s) recognizing these mutants, we used a redesigned reporter, pMV_CAT<sub>am9</sub> <i>metU<sub>CUA/G72G73</sub></i> (Fig. 1f), which is more amenable to Edman degradation. The CAT<sub>am9</sub> reporter consists of an amber codon at position 9 and is preceded by a stretch of six histidine residues to facilitate purification (Fig. S8). Edman degradation (Fig. 5) showed that the amino acid inserted at position 9 was exclusively glutamine, indicating a role for ND-GluRS and GatCAB in recognition of the acceptor stem mutant tRNAs.

**Differential recognition of the acceptor stem mutants by GatCAB.** Our acid urea gel analysis shows that the <i>metU<sub>CUA/G72G73</sub></i> tRNA migrates in two distinct bands (Fig. 4b, lane 9). The slow-migrating band (labeled Q) is labile to both mild and strong alkali treatments (lanes 8 and 7, respectively), suggesting that it corresponds to the aminoacylated form of <i>metU<sub>CUA/G72G73</sub></i>. Furthermore, based on the Edman degradation data (Fig. 5), it may be concluded that the <i>metU<sub>CUA/G72G73</sub></i> tRNA is aminoacylated with Gln. These observations clearly suggest that the <i>metU<sub>CUA/G72G73</sub></i> tRNA is aminoacylated by ND-GluRS and that the attached Glu is then converted to Gln by GatCAB. Comparison of the structures of the native tRNA<sup>Gln</sup> (which is a substrate for GatCAB) and tRNA<sup>Glu</sup> (Fig. 6a) show that the <i>metU<sub>CUA/G72G73</sub></i> mutant is the mutant most similar to tRNA<sup>Gln</sup>, having both a Watson-Crick base pair at position 1·72 and a G73 discriminator base, indicating why it is a substrate for GatCAB, resulting in the complete conversion of the tRNA-attached Glu to Gln. Interestingly, in the case of <i>metU<sub>CUA/G72G73</sub></i> tRNA also, two bands are seen (Fig. 4b, lane 6). The slower-migrating band (labeled E) is labile to both the mild and strong alkali, labeled E, corresponding to the aminoacylated form, migrates much closer to the decylated band (labeled o). This would be consistent with its recognition by ND-GluRS but without further conversion of the attached Glu to Gln (the extra negative charge on Glu makes it migrate closer to the decylated tRNA). The observation also suggests G73 as an important determinant for GatCAB in mycobacteria. The U1·A72 base pair has been shown to be the primary determinant for recognition of tRNA<sup>Gln</sup> by the <i>Staphylococcus aureus</i> GatCAB amidotransferase (18). The A1 mutant (lanes 1 to 3) has an A1·U72 base pair but lacks the G73 discriminator base. Interestingly, this mutant is poorly recognized by GatCAB, and we observe three bands (labeled o, E, and Q) for the <i>metU<sub>CUA/A1</sub></i> tRNA (lane 3). The two slower-migrating bands (Q and E) correspond to the aminoacylated forms (compare with alkali-treated lanes 1 and 2). The slowest of the bands (lane 6, Q) must correspond to Gln-charged tRNA and the middle one (E) to the Glu-charged tRNA. While we are unable to rule out the absence of A73 as a determinant, the results indicate a role for the 1·72 Watson-Crick pair and/or the G73 base in recognition of tRNA by mycobacterial GatCAB. Interestingly, in lane 2, we see a band (labeled with an asterisk) that remains upon treatment with the mild
alkali (CuSO₄). This band quite likely corresponds to the fGln form of metU CUA/A1 tRNA, which would also explain a reasonably good efficiency of initiation by this mutant (Fig. 4c).

**DISCUSSION**

Our understanding of the mechanism of protein synthesis in Gram-positive bacteria, particularly the slow-growing ones, is in the early stages primarily because of the lack of genetic systems that allow the study of these processes in vivo. Well-characterized in vivo reporter systems have enabled genetic analysis of tRNAs in *E. coli*. Using *M. smegmatis* as a model, we have now developed CAT-based reporter systems to specifically measure initiation and elongation activities of i-tRNA mutants in these bacteria. We show that the metU CUA tRNA is poorly aminoacylated by MetRS and ND-GluRS. However, in the presence of heterologous GlnRS from *E. coli*, the metU CUA tRNA is efficiently aminoacylated by Gln. Importantly, our growth curve analyses showed that expression of *E. coli* GlnRS in *M. smegmatis* is not toxic.

Our studies on acceptor stem mutants of the *M. tuberculosis* i-tRNA provide insights into the requirement of formylation for efficient protein synthesis. Formylation is known to be important for efficient translation in *E. coli*. Deletion of the fmt gene encoding formylase results in a slow-growth phenotype. However, in other bacteria like

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**FIG 6** (a) Comparison of secondary structures of the acceptor stem mutants of initiator tRNA with tRNAGlu and tRNAGln from *M. smegmatis*. (b) Proposed pathways for differential recognition of the acceptor stem mutants by the amidotransferase, GatCAB. The G72G73 mutant is efficiently recognized by the amidotransferase, resulting in the complete conversion of the attached Glu to Gln, while the A1 mutant is poorly recognized, resulting in a mixed population of Gln-metU CUA/A1 and Glu-metU CUA/A1. The G72 mutant is not recognized by the enzyme, resulting in its inability to convert the attached Glu to Gln.
Pseudomonas aeruginosa, deletion of the fmt gene is tolerated well with only moderate growth defects (19). The fmt gene was originally proposed to be essential for growth in M. tuberculosis by three independent groups using transposon mutagenesis (20–22). A more recent study on the essentiality of fmt in mycobacteria showed that although it is not essential for survival of M. smegmatis, it is essential for efficient growth of M. bovis (23). Our results with i-tRNA mutants indicate a crucial role for formylation in determining the efficiency of initiation/growth of M. smegmatis.

Mass spectrometric analysis of the reporter proteins indicated the existence of two parallel pathways for the recognition of metU_{CUA} in vivo—aminoacylation by the weak activity of MetRS on the mutant tRNA and a two-step glutaminylation pathway involving recognition by ND-GluRS and GatCAB. Although the analysis does not allow us to quantitatively assess the fraction of tRNA aminoacylated through each pathway, the migration pattern of the aminoacylated population of metU_{CUA} combined with our inability to sequence the reporter by Edman degradation indicates that the predominant pathway may be through ND-GluRS.

Given that overexpression of MetRS in the E. coli CAT_{am1} reporter system increases initiation by metU_{CUA} tRNA (the equivalent of metU_{CUA}) (24), a residual recognition of the metU_{CUA} tRNA (despite it lacking the major recognition element, the CAU anticodon) by MetRS in mycobacteria is not surprising. The presence of GlnRS, which efficiently recognizes metU_{CUA}, overshadows the activity of MetRS in E. coli. The absence of GlnRS (or other synthetases that efficiently recognize the i-tRNA with CUA anticodon) in mycobacteria most likely leads to detectable levels of aminoacylation of i-tRNA_{CUA} by MetRS.

Further, the use of acceptor stem mutants of the M. tuberculosis i-tRNA in our elongation assay reporter helped us to capture enzymatic intermediates in the two-step conversion of glutamate charged i-tRNA_{CUA} (Glu-metU_{CUA}) to glutamine charged i-tRNA_{CUA} (Gln-metU_{CUA}), providing insights into the determinants of GatCAB on the tRNA (Fig. 6b). Biochemical analysis of the transamidation reaction using tRNA^{Asp} and purified GatCAB have previously led to the establishment of the U1-A72 base pair on tRNA^{Asp} as the major determinant for the transamidase (25). Further, mutational analysis of tRNA^{Gln} from Staphylococcus aureus (18) confirmed the feature to be conserved in tRNA^{Gln} as well. The U1-A72 base pair is also conserved in mycobacterial tRNA^{Gln} and tRNA^{Asn}, indicating that the modes of recognition might be similar.

The G1-C72 base pair present in almost all bacterial aspartate and glutamate tRNAs is thought to be a negative determinant for the transamidation reaction. Interestingly, the corresponding base pair on M. tuberculosis tRNA^{Glu} is G1-U72, a feature that is conserved in most members of the order Actinomycetales, including Streptomyces, Nocardia, Rhodobacter, and Corynebacterium. This hints at a possibly unique mechanism of recognition by GatCAB in this class of bacteria. Further, the ability of the G72G73 mutant tRNA to be transamidated completely, in comparison to the G72 mutant tRNA (which has no detectable levels of the transamidated product), indicates a previously uncharacterized role for the discriminator base in tRNA recognition by GatCAB. While our studies do not rule out the absence of A73 as a determinant, it is reasonable to hypothesize an important role for the discriminator base in tRNA recognition, a conclusion supported by the conservation of this base in all species of the Mycobacterium genus (Fig. S9).

Previous studies have shown that while U1 alone is sufficient for converting tRNA^{Asp} into a substrate for GatCAB in Neisseria, A72 seems to play a more prominent role in other bacteria such as Thermus thermophilus (25). The ability of the A1 mutant tRNA (which has an A1-U72 base pair) to become partially transamidated, in comparison to either the wild type (C1-U72 base pair) or the G72 mutant (C1-G72 base pair), indicates a role for the base pair at position 1-72 in tRNA recognition in mycobacteria, in agreement with the previous reports. However, further mutational analysis of the acceptor stem is required to establish the precise determinants of GatCAB and the role of the 1-72 base pair in tRNA recognition by GatCAB in mycobacteria.

To the best of our knowledge, this is the first suppressor tRNA system utilizing native tRNAs to be developed in any species of mycobacteria and has potential implications for the site-specific incorporation of unnatural amino acids into proteins. In spite of the
conserved nature of the protein synthesis machinery across bacteria, the current study provides insights into variations in the common theme across different bacterial classes. The study also complements the insights obtained from in vitro translation systems developed for mycobacteria.

In conclusion, we have established assay systems for both the initiation and elongation steps of protein synthesis in *M. smegmatis*. These assay systems would be helpful in further studies of the mechanism of protein synthesis in mycobacteria.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Strains used are listed in Table 1. *E. coli* strains were grown in LB (containing 1.6% agar for growth on solid media), components of which were procured from BD Difco, NJ. Media were supplemented with ampicillin (100 µg/ml), kanamycin (25 µg/ml), tetracycline (7.5 µg/ml), and chloramphenicol (30 µg/ml) as needed. *M. smegmatis* mc²155 was grown in LB containing 0.2% Tween 80. Media for mycobacteria were supplemented with kanamycin (50 mcg/ml), hygromycin (150 mcg/ml), or chloramphenicol (30 to 120 mcg/ml) as required. Electroporation-mediated transformation of *M. smegmatis* was performed as described previously (26).

**Generation of plasmid constructs.** Plasmids used are listed in Table 1.

(i) **pMV_CAT**<sub>am1</sub>**metU<sub>CUA</sub>**. The CAT<sub>am1</sub> gene (0.8 kb) driven by the *M. tuberculosis* metU gene promoter was obtained by partial digestion of pTK CAT<sub>am1</sub>, a derivative of pTK mtCAT (8), with EcoRI and HindIII and subcloned between the EcoRI and HindIII sites of pMV261.kan (14) to obtain pMV_CAT<sub>am1</sub> (see Fig. S10a in the supplemental material). The CUA anticodon-containing mutant of the *M. tuberculosis* i-tRNA gene (along with its native promoter) was PCR amplified from pTZ mtmetU<sub>CUA</sub> as a derivative of pTZ mtmetU<sub>CUG</sub> (9), using the M13/pUC universal sequencing forward primer and MttrRNA<sub>am1</sub>R, digested with HindIII, and cloned into pMV_CAT<sub>am1</sub> at the HindIII site to obtain pMV_CAT<sub>am1</sub>metU<sub>CUA</sub> (Fig. S10b).

(ii) **Acceptor stem mutants of metU<sub>CUA</sub>**. Acceptor stem mutations in metU were generated by inverse PCR using pMV_CAT<sub>am1</sub>metU<sub>CUA</sub> as the template and metU<sub>G27G73</sub>fp and metU<sub>G27G73</sub>tp, metU<sub>G72</sub>fp and metU<sub>G72</sub>tp, or metU<sub>A1</sub>fp and metU<sub>A1</sub>tp primers. Following 18 cycles of extension by Phusion DNA polymerase, the reactions were subjected to DpnI digestion for 12 h at 37°C and transformed into *E. coli* TG1, and the mutants were verified by DNA sequencing.

(iii) **pMV_CAT**<sub>am27</sub>. The CAT<sub>am27</sub> open reading frame (ORF) was PCR amplified from pRSVCAT<sub>am27</sub>, or pRSV<i>GluRS</i> integrated into the L5<i>att</i> site in *E. coli* 8<i>thi</i>-<i>supE</i> strain as the template and CAT<i>_G72_G73</i>rp, or CAT<i>_G72</i>rp primers. The CAT<sub>am27</sub> gene was released as 260-bp fragments by HindIII digestion of the corresponding pMV_CAT<sub>am27</sub>metU<sub>CUA</sub> constructs and cloned into the HindIII site of pMV_CAT<sub>am27</sub> to obtain pMV_CAT<sub>am27</sub>metU<sub>CUA</sub> (Fig. S10c).

(iv) **pMV_CAT**<sub>am1-His</sub>. The CAT<sub>am1-His</sub> reporter construct was generated by inserting a His tag by site-directed mutagenesis of pMV_CAT<sub>am1</sub>metU<sub>CUA</sub> using CAT<sub>am1-His</sub>fp and CAT<sub>am1-His</sub>rp to insert a His tag downstream of the
amber codon. The PCR product was digested with DpnI for 12 h and introduced into *E. coli* TG1 by transformation. The desired construct was verified by DNA sequencing.

**(vi) pMV_EcoGlnRS.** The ~100-bp *M. tuberculosis* metU promoter was cloned between the EcoRI and BamHI sites of pTKmx (where the xylE gene was deleted by digestion with BamHI and SpII, blunted by Klenow polymerase, and circularized to regenerate the BamHI site), to obtain pTKmx-pmetU. The *E. coli* GlnRS gene was PCR amplified from pACGS (12) using GlnRS_fp and GlnRS_rp primers and ligated to the BamHI site of pTKmx-pmetU to obtain pTKmx-QS. The GlnRS gene along with the metU gene promoter was excised as a 1.9-kb fragment by partial digestion with EcoRI and HindIII (the EcoRI site was blunted using Klenow polymerase) and cloned into the PuILL-HindIII site of pMV361 (Hyg) vector (where the hsp promoter has been deleted by BsrDI digestion) to obtain pMV_EcoGlnRS (Fig. S10d).

**(vii) pET14b_MtuGluRS.** The *M. tuberculosis* gene encoding the non-discriminating GluRS (Rv2992c, gltS [previously gltX]) was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using the primers MtugltX_Ndel_fp and MtugltX_BamHI_rp. The amplicon of 1.5 kb was digested with Ndel and BamHI and cloned into a similarly digested pET14b vector to obtain pET14b_MtuGluRS, which encodes an N-terminally His-tagged version of MtuGluRS.

**(viii) pMV_MtuGluRS.** The gene encoding MtuGluRS was PCR amplified from *M. tuberculosis* H37Rv gDNA using the primers MtugltX_HindIII_fp and MtugltX_Hpal_rp. The 1.5-kb amplicon obtained was digested with Hpal and HindIII and ligated into a similarly digested pMV361 (Hyg') to obtain pMV361 (Hyg') MtuGluRS.

**Growth curve analysis.** Growth analysis was done using a BioScreen C growth analyzer in 100-well honeycomb plates. Hundredfold dilutions of saturated cultures of the strains in five biological replicates were grown in LB-Tween containing 0.1% bovine serum albumin (BSA) at 37°C to saturation, and the optical density at 600 nm (OD$_{600}$) was measured at 3-h intervals. Growth curves were plotted using GraphPad Prism v6 with standard deviation.

**CAT assays.** *M. smegmatis* mc²155 cells harboring the reporter constructs were grown to saturation in LB-Tween and streaked on LB-Tween agar plates containing 30 to 120 μg/ml chloramphenicol. Growth was assessed after 3 days at 37°C. For CAT activity assays, cell-free lysates of the reporter-expressing strains at ~0.7 OD$_{600}$ were prepared by bead-beating in TME buffer (25 mM Tris-HCl [pH 8], 2 mM β-mercaptoethanol, 1 mM Na$_2$ EDTA). CAT assays were performed in 30 μl reaction mixtures consisting (per reaction) of 500 mM Tris-HCl (pH 8.0), 432 μM acetyl coenzyme A (acetyl-CoA), 5,000 pmol cold chloramphenicol, 0.5 μl [¹⁴C]chloramphenicol (55 mCi/mmol, 0.1 μCi/ml), and variable amounts of the cell extracts. Reactions were performed in five replicates with incubations at 37°C for 20 min and extracted with 300 μl ethyl acetate to stop the reactions. The ethyl acetate phase was dried, spotted on silica thin-layer chromatography (TLC) plates, and resolved using a mixture of chloroform and methanol (142:5:7.5) as the mobile phase. Spots were visualized by exposure to phosphorimager screens and scanned on a GE Typhoon FLA-9500 instrument. The percentage of chloramphenicol converted into the acetylated form was calculated densitometrically as the sum of the 1-acetyl and 3-acetyl forms divided by the total chloramphenicol. The percentage of cold chloramphenicol converted was calculated by assuming a conversion ratio similar to that for [¹⁴C]-labeled chloramphenicol. CAT activity was plotted as picomoles of chloramphenicol converted per microgram of total cell proteins used for the assay.

**Immunoblots.** About 30 μg of cell extracts was resolved on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE) using a semidry transfer apparatus (Bio-Rad) in Tris-glycine buffer containing 10% methanol. The membrane was blocked with 5% skim milk for 1 h at room temperature and probed at room temperature for 2 h with a 1:5,000 dilution of anti-CAT antibody (anti-rabbit IgG–alkaline phosphatase) was added at a concentration of 1:5,000. The membrane was washed and stained with 4-nitroblue tetrazolium and was developed colorimetrically using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

**RNA isolation and Northern blot analysis.** Total tRNA was isolated from 5 ml of *M. smegmatis* culture grown in LB-Tween to an OD$_{600}$ of ~0.7. The culture was chilled on ice for 5 min and harvested, and the cells were washed and resuspended in 750 μl SE buffer (0.3 M sodium acetate, 10 mM Na$_2$ EDTA, pH 4.5). An equal volume of SE-saturated phenol was added to the cells along with 200 μl of 0.1-mm-diameter zirconia-silica beads (BioSpec, Inc.). The cells were lysed by bead beating for 4 cycles of 30 s each with a 1-min gap between cycles and extracted with SE-saturated phenol and precipitated with 2.5 volumes of ethanol. RNA was dried, resuspended in 10 mM sodium acetate containing 1 mM Na$_2$ EDTA, and stored at −80°C until required.

Acid urea PAGE, followed by Northern blot analysis, was performed essentially as described previously (15) except that the urea concentration was kept at 6 M. Briefly, about 0.25 OD$_{600}$ of total RNA was mixed with an equal volume of acid urea dye (0.1 M sodium acetate [pH 5.0], 8 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol) and resolved on a 6 M acid urea PAGE gel (6.5%) at 500 V (~20 V/cm) in 0.1 sodium acetate, pH 5.0, until the bromophenol blue dye reached the bottom of the gel. The region between the two dyes was blotted onto a positively charged nylon membrane (Hybond-XL, GE) using Tris-borate-EDTA (TBE) as the transfer buffer, UV cross-linked at 1,200 V/cm (500 W) for 1 h at 4°C. Transfer was monitored by exposure to a UV transilluminator. The blots were exposed to phosphorimagery screens, and scanned with a GE Typhoon FLA-9500 instrument.
TABLE 2 List of DNA oligomers used in the study

<table>
<thead>
<tr>
<th>DNA oligomer</th>
<th>Sequence (5′−3′)</th>
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<tr>
<td>M13/pUC universal sequencing primer (−40)</td>
<td>TATCCAGTGGTTTTTTTGTCCTACCCATGGTGGTGGTGGTGCATTTTTAGCTTCCTTTAGCTCC</td>
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<tr>
<td>MtrRNArp</td>
<td>AGCAAAAGCTTAAAAAAAATTACGCCCC</td>
</tr>
<tr>
<td>metU_G72G73fp</td>
<td>CGGGATCCACACCAACCACCAACCACGAAAGCTATAGGAGAAGAAAAAAACACTGG</td>
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<tr>
<td>metU_G72G73rp</td>
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<td>metU_G72fp</td>
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<tr>
<td>metU_G72rp</td>
<td>TATAGGATCTACGCCGTTATATACG</td>
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<tr>
<td>metU_A1fp</td>
<td>AGCGGGATCCACACCAACCACCAACCACGAAAGCTATAGGAGAAGAAAAAAACACTGG</td>
</tr>
<tr>
<td>metU_A1rp</td>
<td>AACCGGGATCCACACCAACCACCAACCACGAAAGCTATAGGAGAAGAAAAAAACACTGG</td>
</tr>
<tr>
<td>HisCAT_BamHI_fp</td>
<td>CCGGGATCCACACCAACCACCAACCACGAAAGCTATAGGAGAAGAAAAAAACACTGG</td>
</tr>
<tr>
<td>CAT_HindIII_fp</td>
<td>AAACAGCTTTTTTTTTTTGGTATACGCGCGCGG</td>
</tr>
<tr>
<td>CAT_am1_fp</td>
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<td>CAT_am1_rp</td>
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<tr>
<td>CAT_am1-His_fp</td>
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<td>CAT_am1-His_rp</td>
<td>CCGGGATCCACACCAACCACCAACCACGAAAGCTATAGGAGAAGAAAAAAACACTGG</td>
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<tr>
<td>GlnRS_fp</td>
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<td>GlnRS_rP</td>
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<td>MtuGluTX_Ndel_fp</td>
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<td>MtuGluTX_BamHI_fp</td>
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<td>MtuGluTX_HindIII_fp</td>
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<td>MtuGluTX_Hpal_rp</td>
<td>ACAGTTAACTACCCGTCGCCCC</td>
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Purification of MtuGluRS and activity assays. An N-terminally His-tagged version of MtuGluRS in the vector pET114b was expressed in E. coli BL21(DE3) Rosetta pLysS cells and the protein was purified by Ni-NTA affinity chromatography. Briefly, cultures were grown at 30°C to an OD_{600} of 0.4, and expression of the protein was induced at 30°C using 0.05 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. The cells were harvested and lysed by sonication in buffer A (20 mM Tris-HCl [pH 8], 500 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole, 10% glycerol). The supernatant was clarified by ultracentrifugation at 100,000 × g and passed through a 5-mL Ni-NTA column (GE). The column was washed with 10 volumes of buffer A, and the protein was eluted with a step gradient of 50 mM, 100 mM, 200 mM, 300 mM, 500 mM, and 1,000 mM imidazole in buffer A. Protein-containing fractions were pooled, dialyzed against buffer A containing 50% glycerol. Other aminoacyl-tRNA synthetases were purified using a similar method.

Aminoacylation assays were performed in a 10-μL reaction mixture containing 55 mM HEPES/NaOH (pH 7.3), 25 mM KCl, 2 mM dithiothreitol (DTT), 10 mM MgCl₂, 1 mM ATP, 0.1% BSA, 2 mM amino acids, and 0.1 OD_{600} total tRNA isolated from M. smegmatis mc²155 expressing M. tuberculosis metU_{mc²}, or its mutants, and 1 μg of purified aminoacyl-tRNA synthetases. Reactions were performed at 37°C for 30 min, and the contents were supplemented with an equal amount of acid urea dye and resolved on a 6 M 6.5% acid urea PAGE gel. Northern blot analysis was performed using probes specific to the tRNA of interest.

Purification of reporter proteins and Edman degradation. Reporter proteins were enriched from M. smegmatis cells overexpressing either pMV_CAT_{am1}, metU_{CAGGCGTGCGCG} or pMV_CAT_{am1}, metU_{CAGGCGTGCGCG} by Ni-NTA affinity chromatography as described above. In the case of CAT_{am1}, the reporter protein was purified with or without coexpression of EcoGlnRS. For Edman degradation, about 100 pmol of the purified protein was blotted onto a PVDF membrane using borate buffer (50 mM borate, 10% methanol [pH 9.0], adjusted with NaOH). The region of the blot corresponding to CAT was excised, washed extensively with water, and submitted for protein sequencing at the Molecular Structure Facility, University of California, Davis, CA.

Mass spectrometric analysis. Enriched fractions of CAT_{am1}, purified with or without coexpression of EcoGlnRS, were resolved on a 12% SDS-PAGE gel and the band corresponding to CAT was subjected to in-gel trypsin digestion. Briefly, the bands were excised, washed thoroughly with water, destained with 50% acetonitrile–10 mM ammonium bicarbonate solution, dehydrated with 100% acetonitrile, vacuum dried, and subjected to trypsin digestion at 37°C for 18 to 24 h. The resulting peptides were collected by two extractions with 50% acetonitrile and one extraction with 100% acetonitrile. The pooled peptides were vacuum dried to remove organic solvents and resuspended in 500 μL of 50 mM Tris-HCl (pH 7.5). Ni-NTA resin (20 μL) was added to the mixture, and the peptides were allowed to bind at 4°C for 3 h. The beads were washed three times with 10 mM imidazole in 50 mM Tris-HCl (pH 7.5), and the peptides were eluted in 50 mM Na₂ EDTA. The peptides were desalted using a 0.2-μL C₁₈ Zip-Tip (Millipore) to remove Na₂ EDTA and subjected to matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) analysis. Spectra were acquired in the positive mode using α-cyano-cinnamic acid (CCA) as the matrix in a Bruker Daltonics Ultraflex MALDI-TOF instrument. Peaks of interest were subjected to MS/MS by LIFT-mediated fragmentation. Spectra were annotated manually using Flex Analysis v3.2.
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REFERENCES


