

Sustenance of *Escherichia coli* on a single tRNA^{Met}

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ABSTRACT

Living organisms possess two types of tRNAs for methionine. Initiator tRNAs bind directly into the ribosomal P-site to initiate protein synthesis, and the elongators bind to the A-site during the elongation step. Eubacterial initiators (tRNA^{fMet}) are unique in that the methionine attached to them is formylated to facilitate their binding to initiation factor 2 (IF2), and to preclude them from binding to elongation factor Tu (EFTu). However, in mammalian mitochondria, protein synthesis proceeds with a single dual function tRNA^{Met}. *Escherichia coli* possesses four tRNA^{fMet} (initiator) and two tRNA^{Met} (elongator) genes. Free-living organisms possessing the mitochondrion like system of single tRNA^{Met} are unknown. We characterized mutants of *E. coli* tRNA^{fMet} that function both as initiators and elongators. We show that some of the tRNA^{fMet} mutants sustain *E. coli* lacking all four tRNA^{fMet} and both tRNA^{Met} genes, providing a basis for natural occurrence of mitochondria like situation in free living organisms. The tRNA mutants show *in vivo* binding to both IF2 and EFTu, indicating how they carry out these otherwise mutually exclusive functions by precise regulation of their *in vivo* formylation. Our results provide insights into how distinct initiator and elongator methionine tRNAs might have evolved from a single ‘dual function’ tRNA.

INTRODUCTION

Living organisms have evolved with two essential and distinct forms of methionine decoding tRNAs. The initiator tRNA (i-tRNA or tRNA^{fMet}) inserts methionine at the first position, and the elongator tRNAs (tRNA^{Met}) at the downstream positions in proteins (1). In bacteria, while tRNA^{fMet} is recognized by initiation factor 2 (IF2) for its binding to the ribosomal P-site, tRNA^{Met} binds elongation factor Tu (EFTu) to enter the A-site (2,3). The exclusive role of tRNA^{fMet} in initiation is facilitated by its unique structural and sequence characteristics (4). The three consecutive G:C base pairs found in the anticodon stems of i-tRNAs are

evolutionarily conserved in all domains of life, and facilitate i-tRNA binding to the ribosomal P-site. In addition, tRNA^{fMet} possesses a Watson Crick mismatch at position 1 × 72, which together with the 2:71 and 3:70 base pairs (Supplementary Figure S1), constitutes a major element for its recognition by methionine-tRNA formyltransferase (Fmt) (5,6). Formylation of the amino acid attached to tRNA^{fMet} plays a critical role in its binding to IF2 (3,7,8).

In contrast, mammalian mitochondria possess a single tRNA^{Met} (9) which binds to the mitochondrial IF2 (IF2_{mt}) to take part in initiation (when formylated), and to EFTu_{mt} to take part in elongation (when not formylated). Competition between Fmt_{mt} and EFTu_{mt} for binding to Met-tRNA^{Met} is assumed to ensure the dual function of the tRNA^{Met} in mammalian mitochondria (10). The dual-nature of this tRNA^{Met} is aided, at least in part, by the fact that Fmt_{mt} primarily recognizes the amino acid attached to the tRNA as opposed to eubacterial Fmt, whose primary determinants are situated in the acceptor stem of the tRNA itself, necessitating two distinct tRNAs for initiation and elongation functions (10). In yet another example of dual function tRNAs, *Trypanosoma brucei*, a protist that lacks mitochondrially encoded tRNAs and relies on tRNAs imported from the cytosol to sustain translation in the organelle, utilizes the eukaryotic-type elongator tRNA^{Met} imported from cytosol for initiation after its formylation in mitochondria (11). Understanding the origin of dedicated tRNAs for initiation and elongation from such dual function tRNAs may provide evolutionary insights. In addition, it is of interest to understand the mechanism by which the single mitochondrial tRNA^{Met} is distributed into the initiation and elongation steps of protein synthesis.

In *E. coli*, of the four tRNA^{fMet} genes, three (*metZ*, *metW* and *metV*) encoding tRNA^{fMetI} are in an operon at 63.5 min (*metZ WV*), and the fourth (*metY*) encoding tRNA^{fMetII} is present at 71.49 min (Figure 1A and B). The tRNA^{fMetI} and tRNA^{fMetII} differ by a single nucleotide at position 46 (12). Interestingly, this change allows separation of the two tRNAs on the native gels (13). As for the elongators, two genes (*metT* and *metU*) encoding identical tRNA^{Met} are present in the *metT-leuW-glnU-glnW-metU-glnV-glnX* operon at 15.02 min along with other essential elongator tRNA genes (Figure 1C). The *metZ WV* and

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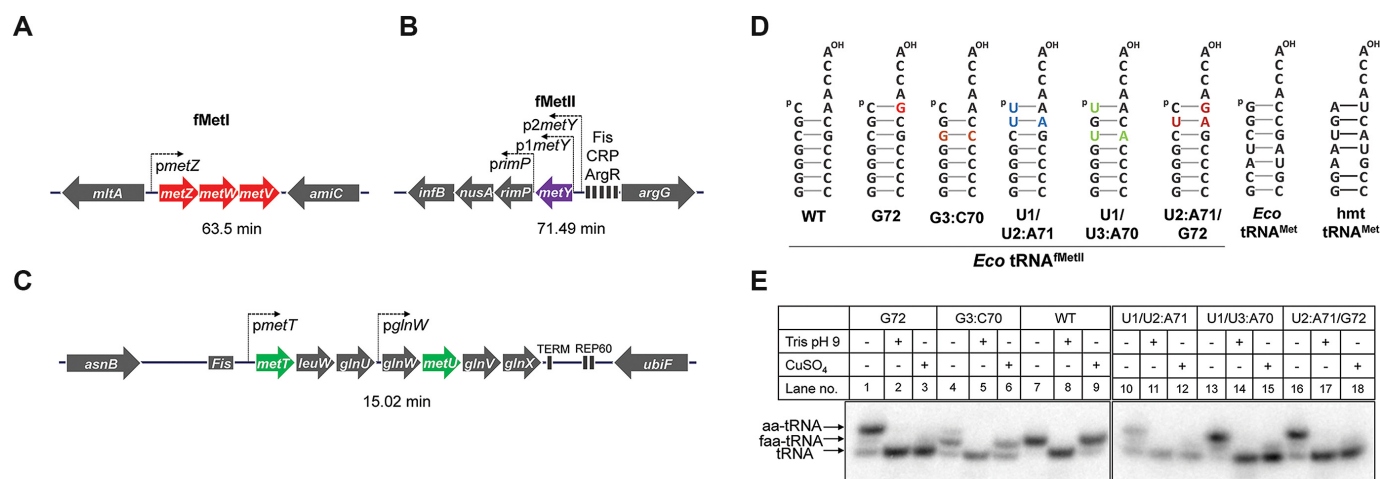


Figure 1. Mutants of the *E. coli* initiator tRNA and their *in vivo* status. Schematic of organization of the initiator (A and B) and elongator (C) methionine tRNA gene loci in *E. coli*. fMetI encoding tRNA genes (*metZ**W**V*) are shown in red, the fMetII encoding gene (*metY*) is shown in purple and the methionine elongator tRNA genes (*metT* and *metU*) are shown in green. Only the promoters relevant to tRNA gene transcription have been depicted. (D) Structures of the acceptor stems of the *E. coli* tRNA^{fMet}, tRNA^{fMet} mutants used, and tRNA^{Met}. Mutations are shown in colour. (E) *In vivo* status of the tRNA^{fMet} mutants as analyzed by acid urea PAGE followed by northern blot analysis using ³²P-5'-end labeled oligomers complementary to nucleotides 39–55 of *metY*. Total tRNA was isolated from Δ *metY* strains overexpressing the mutant tRNAs from plasmid. Aminoacylated tRNA population is depicted as aa-tRNA, formylated tRNA population as faa-tRNA and decylated tRNA population as tRNA.

metY genes can be independently deleted without significantly affecting growth at 37°C (14–16) (Supplementary Figure S2). Because the function of tRNA^{fMet} is essential in *E. coli*, simultaneous deletion of both loci can only be achieved in the presence of supporting tRNA^{fMet} gene(s) elsewhere in the chromosome or on a plasmid (16). Likewise, it should be possible to delete both the chromosomal copies of the tRNA^{Met} genes in the presence of tRNA^{Met} gene support. However, generating a mitochondrion like system for tRNA^{Met} in *E. coli* would demand deletion of all four of the tRNA^{fMet} (*metZ**W**V* and *metY*) and both the tRNA^{Met} (*metT* and *metU*) genes from the chromosome and sustaining the strain on a single gene encoding methionine tRNA with dual functions as initiator and elongator.

MATERIALS AND METHODS

Strains, plasmids and DNA oligomers

Lists of strains/plasmids and DNA oligomers used are provided in Supplementary Tables S1 and S2.

Generation of plasmid constructs

pT6. The 881 bp genomic region containing the *leuW-glnU-glnW-metU-glnV-glnX* operon was PCR amplified from *E. coli* KL16 genomic DNA using the primers *leuW*_NcoI_{fp} and *glnX*_HindIII_{rp}. The amplicon was digested with NcoI and HindIII and ligated into similarly digested pACDH (Tet^R) to obtain pT6. Expression of the tRNAs from the construct was verified by northern blot analysis (Supplementary Figure S3).

pA5. The regions containing the genes *leuW-glnU-glnW* and *glnV-glnX* were PCR amplified from *E. coli* KL16 genomic DNA using the primers *leuW*_NcoI_{fp},

*glnW*_EcoRI_{rp}, *glnV*_EcoRI_{fp} and *glnX*_HindIII_{rp} respectively. The PCR amplicons were sequentially ligated into the NcoI EcoRI and EcoRI HindIII sites in pACDH (Tet^R) to obtain pT5. The Tet marker in pT5 was then disrupted by NruI EcoRV digestion and a blunt ended 1369 bp ampicillin resistance (Amp^R) cassette obtained by SnaBI/MscI digestion of pKD4 was ligated at this site to obtain pA5. The resulting plasmid contains 5 genes of the 7 gene operon excluding *metT* and *metU*. The constructs were verified by restriction digestion followed by DNA sequencing.

pIF2 and *pEFTu*. The genes encoding IF2 and EFTu (*infB* and *tufA*) were PCR amplified from *E. coli* KL16 genomic DNA with the primers IF2_His_{fp}, IF2_{rp}, EFTu_{fp} and EFTu_His_{rp} designed to incorporate N-terminal and C-terminal His tags, respectively. The amplicons of 2.7 and 1.2 kb, respectively were cloned into NcoI/HindIII sites of pA5, replacing the tRNA genes in the process. The clones were screened by restriction digestion and confirmed by DNA sequencing.

Generation of strains

Δ *metZ**W**V*::*cm*. *E. coli* KL16/pKD46 was grown at 30°C to 0.2 OD₆₀₀, induced with 1 mM arabinose and made electrocompetent at 0.7 OD₆₀₀ by three successive washes in sterile 10% glycerol, obtaining a 100-fold concentration of cells in the process. A 50 μ l aliquot of the cells was used for electroporation. The linear DNA substrate for recombination was made by PCR amplification of the chloramphenicol (cm)^R cassette from pKD3 with 36 bp flank sequences corresponding to the *metZ**W**V* locus using the primers *metZ**W**V*_Kof_p and *metZ**W**V*_Kor_p. About 300 ng of the 1 kb amplicon was electroporated into the cells at 1.8 kV, 200 Ω and 25 μ F in a 0.1 cm cuvette. The cells were allowed to

recover in LB for 4 h at 37°C and selected on LB agar containing 30 µg/ml chloramphenicol. Knockouts were confirmed by colony PCR with *metZWW*_fp and *metZWW*_rp where knockout strains give an amplicon of 1087 bp, while the parental strain gives an amplicon of 331 bp (Supplementary Figure S4). The knockout strains were further confirmed by native PAGE of total tRNA followed by northern blot analysis to verify the absence of tRNAs derived from the *metZWW* locus (Supplementary Figure S2).

Δ metY. An *E. coli* KL16 strain with the *metY* gene replaced by a chloramphenicol cassette, Δ metY::cm, has been reported previously (16). The chloramphenicol cassette used to replace *metY* was removed by pCP20 mediated expression of Flp recombinase to make an unmarked deletion of *metY*.

Δ 7::kan/pT6. The strain Δ 7::kan/pT6 where the entire operon of the 7 tRNA genes (*metT-leuW-glnU-glnW-metU-glnV-glnX*) is deleted and supported by six of the genes (excluding *metT*) on a plasmid copy was generated by lambda Red mediated recombination. The knockout cassette was PCR amplified using pKD4 as template with the primers *metT_KO*fp2 and *metT_KO*rp2. The 1571 bp PCR product was electroporated into *E. coli* KL16/pT6/pKD46 as above. Selection was carried out on LB agar containing 25 µg/ml kanamycin and the knockout strains were confirmed by colony PCR using *metT_KO*.conf.fp and *metT_KO*.conf.rp where knockout strains give a 2 kb amplicon while the parental strain gives a 1 kb amplicon (Supplementary Figure S5).

Strains sustained on mutant tRNAs for initiator function

Strains deleted for all four genomic copies of the initiator tRNA and supported by mutant tRNAs on plasmid (Δ metZWW::cm Δ metY/pmetY*; also referred to as DK/pmetY*) were generated by P1 phage transduction mediated transfer of the Δ metZWW::cm locus from *E. coli* KL16 Δ metZWW::cm to Δ metY/pmetY*. Strains were selected on LB agar containing 30 µg/ml chloramphenicol and 7.5 µg/ml tetracycline. Transductants were verified by colony PCR using *metZWW*_fp and *metZWW*_rp as above. Deletion of all wild type initiator tRNA gene copies was further verified by native PAGE of total tRNA followed by northern blot analysis using the met33 probe, which binds to initiator tRNA molecules derived from both *metZWW* and *metY* locus. Reversion of the mutant tRNA genes supplied on plasmid to the wild type sequence was ruled out by DNA sequencing of the plasmid isolated from the knockout strains.

Strains sustained on mutant tRNAs for elongator tRNA^{Met} function

Strains deleted for both copies of elongator tRNA^{Met} (Δ 7::kan Δ metY::cm/pmetY*/pA5; renamed as EK/pmetY*) were generated by P1 transduction mediated transfer of the Δ 7::kan locus from Δ 7::kan/pT6 strain to Δ metY::cm/pA5/pmetY* strain. Strains were

selected on LB agar containing 25 µg/ml kanamycin, 100 µg/ml ampicillin and 7.5 µg/ml tetracycline. Transductants were verified by colony PCR using *metT_KO*.conf.fp and *metT_KO*.conf.rp as above. Deletion of all elongator tRNA^{Met} gene copies was further verified by native PAGE of total tRNA followed by northern blot analysis using met-elongator probe. Reversion of the mutant tRNAs to the wild type sequence was ruled out by DNA sequencing of the plasmid isolated from the knockout strains.

Strains sustained on mutant tRNA^{fMet} for initiator and elongator functions

Strains deleted for all copies of the native initiator and elongator methionine tRNA genes (Δ metZWW::cm Δ metY Δ 7::kan/pmetY*/pA5; referred to as TK/pmetY*) were generated using the Δ metZWW::cm Δ metY strain constructed as above. The double knockout strains harboring pA5 were transduced with P1 phage raised on Δ 7::kan/pT6. The resulting transductants are deleted for all genomic copies of methionine initiator and elongator tRNAs and are sustained exclusively on mutant tRNA^{fMet} supplied on plasmid. Transductants were selected on LB agar containing 100 µg/ml ampicillin, 25 µg/ml kanamycin and 7.5 µg/ml tetracycline and were verified by colony PCR using *metT_KO*.conf.fp and *metT_KO*.conf.rp as above. Deletion of all elongator and initiator methionine tRNA gene copies was further verified by native PAGE of total tRNA followed by northern blot analysis using both met33 and met-elongator probes. Reversion of the mutant tRNAs to the wild type sequence was ruled out by DNA sequencing of the plasmid isolated from the knockout strains.

Δ metY tufB-His:kan

EFTu is encoded by two near-identical genes, *tufA* and *tufB* in *E. coli*. A C-terminal His-tag was added to the genomic copy of *tufB* by lambda Red mediated recombination. A 1.25 kb kanamycin cassette was PCR amplified from pST-K (17) using the primers Kan_HindIIIfp and Kan_HindIIIrp and cloned downstream of the EFTu gene at the HindIII site in pEFTu to obtain pEFTu-K. The C-terminal region of EFTu including the His-tag and its 100 bp upstream region were PCR amplified along with the kanamycin cassette from pEFTu-K incorporating a 40 bp flank sequence (corresponding to the genomic sequence downstream of *tufB*) at the 3' end using the primers *tufB_KI*_fp and *tufB_KI*_rp. The 1454 bp PCR product was electroporated into KL16/pKD46 cells as above and recombinants were selected on LB agar containing 25 µg/ml kanamycin. The strains were verified by colony PCR using *tufB*_flankfp and *tufB*_flankrp. The *tufB*-His:kan locus was moved into KL16 Δ metY by P1 mediated transduction to obtain KL16 Δ metY *tufB*-His:kan.

Growth analysis and doubling times

Growth analysis was performed in a BioScreen C growth analyzer using 100-well honeycomb plates. Thousand-fold dilutions of the strains in 5 biological replicates were grown

in LB at either 37°C or 22°C to saturation and OD₆₀₀ was measured at 1 h intervals. Growth curves were plotted using GraphPad Prism v6 with SEM. Doubling times were calculated from manual growth curves performed in duplicates. Cells were grown in 100 ml conical flasks containing 20 ml of LB at 37°C and 180 rpm. Aliquots (100 µl) of cells were taken at hourly intervals and OD₆₀₀ was measured. The linear part of the curve was identified by plotting the natural log of OD₆₀₀ against time. Doubling times were calculated from the linear part of the curve using the formula: $d = \ln 2 * (t_2 - t_1) / \ln (OD_2 - OD_1)$, where OD₂ and OD₁ are the OD₆₀₀ values at times t_2 and t_1 .

IF2 and EFTu pulldowns

IF2 and EFTu pulldowns were performed in DK/*pmetY**/pIF2 and either *E. coli* KL16/*pmetY**/pEFTu or KL16 Δ *metY tufB*-His:*kan*, respectively. About 300 ml of each culture was grown to about 1.0 OD₆₀₀ at 37°C without induction. All further steps were performed at 4°C. The cultures were chilled on ice, harvested, washed and resuspended in 4 ml of lysis buffer (20 mM Tris-Cl pH 7.8, 500 mM NaCl, 2 mM β -mercaptoethanol, 1 mM GTP). Cells were incubated for 1 h on ice after addition of 100 µl of 40 mg/ml lysozyme and subsequently lysed by freeze-thaw in liquid nitrogen. The lysate was supplemented with 20 U of RNase-free DNase I and 0.2 mM CaCl₂ during the last 15 min of incubation. The clear supernatant obtained after centrifugation at 20 000g for 30 min at 4°C was mixed with 100 µl Ni-NTA agarose beads (Qiagen) pre-equilibrated with lysis buffer containing 10 mM imidazole. Binding was allowed to occur for 1 h at 4°C with shaking. The beads were harvested by centrifugation at 3000g for 1 min and washed thrice with 1 ml lysis buffer containing 10 mM imidazole. Proteins were eluted from the beads using 300 µl lysis buffer containing 1 M imidazole. About 20 µl of this eluted fraction was loaded on a 12% SDS-PAGE to visualize the pulled down proteins. About 50 µl of the fraction was resolved on a 15% native PAGE to visualize the tRNA bound to IF2 or EFTu. Northern blot analysis was performed using a mixture of ³²P end-labelled met33 and met-elongator probes.

Phenotypic microarray profiling

Phenotypic microarrays were performed using strains freshly streaked out on Biolog Universal Growth (BUG) agar. Briefly, the colonies were resuspended in the manufacturer supplied 'IF0 inoculation fluid' (Biolog Inc., Hayward CA, USA) containing 'Redox dye mix A' to obtain a turbidity of 85% T. Aliquots (100 µl) of this suspension were added into each well of the phenotypic microarray plates PM1 (carbon sources), PM3 (nitrogen sources), or PM5 (nutrient supplements). For PM3 and PM5, 20 mM sodium pyruvate was added as the carbon source. The plates were incubated at 37°C for 48 h and reduction of the dye was monitored. Pairwise comparisons of the growth profiles of strains were performed using the manufacturer supplied software.

Identification of natural tRNAs similar to the dual-function mutants

To identify tRNA genes with characteristics similar to the dual-function mutant tRNAs generated, the list of predicted tRNAs from all sequenced bacterial genomes was downloaded from tRNADB-CE (<http://trna.ie.niigata-u.ac.jp>) (18), a manually curated database of tRNAs predicted by multiple analyses. The sequences corresponding to initiator tRNAs were filtered out and tRNAs containing a 1:72 pair were identified using a regular expression search. The identified sequences were manually verified by determining the most stable secondary structure using RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) (19).

RESULTS

Formylation deficient mutants of the *E. coli* tRNA^{fMet} function as initiators and elongators

Mutants of tRNA^{fMet} harboring substitutions in its acceptor stem (G72, G72G73, G3:C70, U1, U1/U2:A71, U1/U3:A70 and U2:A71/G72) are known, which are capable of functioning as elongators or both as initiators and elongators at least in an *in vitro* translation system or in a reporter based system in *E. coli* (20). These tRNA^{fMet} mutants are defective in formylation (by Fmt) to varying degrees, and have become substrates for peptidyl-tRNA hydrolase (Pth) which hydrolyses formyl-aminoacyl-tRNA^{fMet} (with a Watson:Crick base pair at 1:72) to free tRNA (20–22). However, it is unknown whether these tRNAs are capable of sustaining the entire initiation or elongation load of the cell. Could these tRNAs sustain the cellular demand for all the six genes that encode the initiators (tRNA^{fMet}) and elongators (tRNA^{fMet}) and allow *E. coli* to survive?

Of the above tRNA^{fMet} acceptor stem mutants, the U1 mutant is not significantly compromised for its formylation by Fmt and serves as a poor elongator even in a reporter based assay (20). And, the G72G73 mutant is an extremely poor substrate for Fmt (20). Thus, we excluded both these mutants from our analyses. Analyses of the remaining mutants (G72, G3:C70, U1/U2:A71, U1/U3:A70 and U2:A71/G72, Figure 1D) on acid urea gels for their *in vivo* aminoacylation/formylation revealed their cellular accumulation both in the aminoacylated and formyl-aminoacylated forms (Figure 1E). As a control, the wild type tRNA^{fMet} accumulated in completely formylated form (Figure 1E, lane 7). The G72, U1/U3:A70 and U2:A71/G72 mutants, existed predominantly in aminoacylated form with ~20–30% of the tRNAs present in formylated form (Figure 1E and Supplementary Figure S6). The G3:C70 mutant existed predominantly in the formylated form (Figure 1E, lane 4), and the U1/U2:A71 mutant had higher levels of the aminoacylated form (Figure 1E, lane 10). A small fraction of most tRNA^{fMet} mutants was also present in the deacylated form as, owing to the Watson:Crick base pair at the 1:72 position, they are substrates for Pth (20). The U1/U2:A71 mutant, most likely due to its structural instability (22), accumulates to lower cellular levels than the other mutants. Nonetheless, the analysis en-

dorsed the suitability of the tRNA^{fMet} mutants for further investigation.

Mutants of tRNA^{fMet} that sustain *E. coli* for initiation

To test if the mutant tRNA^{fMet} sustained the entire load of initiation in *E. coli* to allow deletion of all four tRNA^{fMet} genes from the chromosome, we used a transduction based genetic strategy (16) (Supplementary Figure S7a) wherein P1 phage lysate raised on $\Delta metZWW::cm$ donor strain (deleted for the *metZWW* locus) was used to transduce the $\Delta metY$ recipient strains harboring plasmid borne genes for the wild type or mutant tRNA^{fMet} (indicated as *pmetY**). The resulting transductants ($\Delta metZWW::cm \Delta metY/pmetY^*$) referred to as double locus knockout strains (DK/*pmetY**) were deleted for all four tRNA^{fMet} genes (*metZWW* and *metY*) and supported entirely by a plasmid borne tRNA^{fMet} (wild type or mutant) gene (Figure 2A). Growth curve analysis at 37°C (Figure 2B) revealed that the deletion strain supported by wild type tRNA^{fMet} (DK/*pmetY*) grew the best (pink curve). Consistent with the significant fraction of their accumulation in formylated form, the G3:C70 and U1/U3:A70 were most capable of initiation (olive and blue curves). Growth of the strain supported by the U1/U2:A71 mutant (dark green) was comparable to that of the G72 mutant supported strain (orange). The strain supported by the U2:A71/G72 mutant (purple) and which accumulated very little in the formylated form, grew the poorest. All the strains (supported by the mutant tRNA^{fMet}) were cold sensitive at 22°C (Figure 2c) like the $\Delta metZWW$ strain (15,23). Importantly, all the mutants (G72, G3:C70, U1/U2:A71, U1/U3:A70 or U2:A71/G72) or the wild type tRNA^{fMet} are capable of sustaining *E. coli* for the cellular load of initiation. However, we do not discount that in the cases of the mutant tRNAs which are sub-optimal in their function, their increased expression (from the plasmid borne genes) might have also contributed to the sustenance of the strain.

Mutants of tRNA^{fMet} that sustain *E. coli* for elongation

Next, we designed a strategy to test if the tRNA^{fMet} mutants support *E. coli* for its elongator tRNA^{Met} function. Both *metT* and *metU* genes encode identical tRNA^{Met}, and are in the *metT-leuW-glnU-glnW-metU-glnV-glnX* operon with five other essential tRNA genes. Using standard genetic engineering techniques, we first cloned two versions of the operon segments into plasmids, pT6 (*leuW-glnU-glnW-metU-glnV-glnX*) and pA5 (*leuW-glnU-glnW-glnV-glnX*), such as to lack either one (*metT*) or both (*metT* and *metU*) tRNA^{Met} genes, respectively (Supplementary Figure S8). The pT6 (Tet^R) and pA5 (Amp^R) are pACYC based plasmids compatible with *pmetY** (tRNA^{fMet} harboring plasmids). We then followed a two-step protocol. In the first step, we generated $\Delta(metT-leuW-glnU-glnW-metU-glnV-glnX)::kan/pT6$ strain (referred to as $\Delta 7::kan/pT6$), deleted for all seven tRNA genes of the operon from the chromosome with pT6 support (Supplementary Figure S8). The resulting strain has no tRNA^{Met} genes on the chromosome and is sustained by a single tRNA^{Met} gene, *metU*, on pT6. In the second step, we transduced

the $\Delta metY::cm/pA5/pmetY^*$ strain (recipient) with the P1 raised on $\Delta 7::kan/pT6$ (donor) strain (Supplementary Figure S7b). The resulting transductants ($\Delta metY::cm \Delta 7::kan/pA5/pmetY^*$; referred to as elongator knockout strains, EK/*pmetY**) are devoid of all copies of the elongator tRNA^{Met} genes and are sustained for the elongation function entirely by the plasmid borne mutant tRNA^{fMet}. In this experiment, we obtained transductants for the strains harboring G72, U1/U2:A71, U1/U3:A70 and U2:A71/G72 *metY* genes (Figure 2D). However, the strain EK/*pmetY** harboring wild type tRNA^{fMet} gene did not yield transductants, suggesting, not unexpectedly, that it is unable to sustain elongation function. Also, the strain EK/*pmetY** harboring the G3:C70 mutant tRNA^{fMet} gene failed to produce any transductants. In fact, only a minor fraction of the G3:C70 tRNA^{fMet} accumulated in aminoacylated form explaining why this mutant might have failed to substitute for the elongator tRNA^{Met} function. In the growth curve experiment, the strains supported by either of the G72, U1/U2:A71, U1/U3:A70 and U2:A71/G72 mutants grew comparably at 37°C (Figure 2E). However, at 22°C (Figure 2F) the strains supported by G72 (Figure 2f, orange) and U1/U3:A70 (Figure 2F, blue) were the healthiest, followed by U2:A71/G72 (Figure 2F, purple). The strain supported by U1/U2:A71 (Figure 2F, dark green) was highly compromised for growth at 22°C, although it grew at 37°C. These data showed that at least some of the tRNA^{fMet} mutants could sustain *E. coli* for the elongator tRNA^{Met} function. It may be noted that while the EK/*pmetY** strains are deleted for *metY*, they possess the *metZWW* locus encoding three of the four tRNA^{fMet} genes.

Mutants of tRNA^{fMet} that sustain *E. coli* for both initiation and elongation

From the above experiments, we obtained four mutants of tRNA^{fMet} (G72, U1/U2:A71, U1/U3:A70 and U2:A71/G72), which sustained *E. coli* for the independent functions of initiation and elongation. As the next step, we wished to check (by deletion of the *metZWW* locus from the EK/*pmetY** strains) if any of the four tRNA^{fMet} mutants, supported *E. coli* for the tRNA^{fMet} and tRNA^{Met} functions, simultaneously. To do this, we transduced the double knockout strains (DK/*pA5/pmetY**) supported by any of the four tRNA^{fMet} mutants with the P1 raised on the $\Delta 7::kan$ strain to obtain triple locus knockout (TK) strains deleted for all copies of the chromosomal tRNA^{fMet} and tRNA^{Met} genes ($\Delta metY \Delta metZWW::cm \Delta 7::kan/pA5/pmetY^*$ (or TK/*pmetY**) (Supplementary Figure S7c). Interestingly, we were able to obtain triple knockout strains with three of the tRNA^{fMet} mutants G72, U1/U2:A71 and U1/U3:A70 (Figure 2G). Under the conditions used, the U2:A71/G72 mutant did not support a triple knockout even though it supports independent functions of either initiation or elongation. The strains grew comparably at 37°C (Figure 2H) or at 22°C (Figure 2I), with the strain supported by U1/U3:A70 tRNA^{fMet} growing slightly better than the other two mutants.

To characterize the knockout strains further, we assessed the growth of KL16 (parent strain), $\Delta metY$, $\Delta metZWW::cm$, DK/U1/U3:A70, TK/G72 and

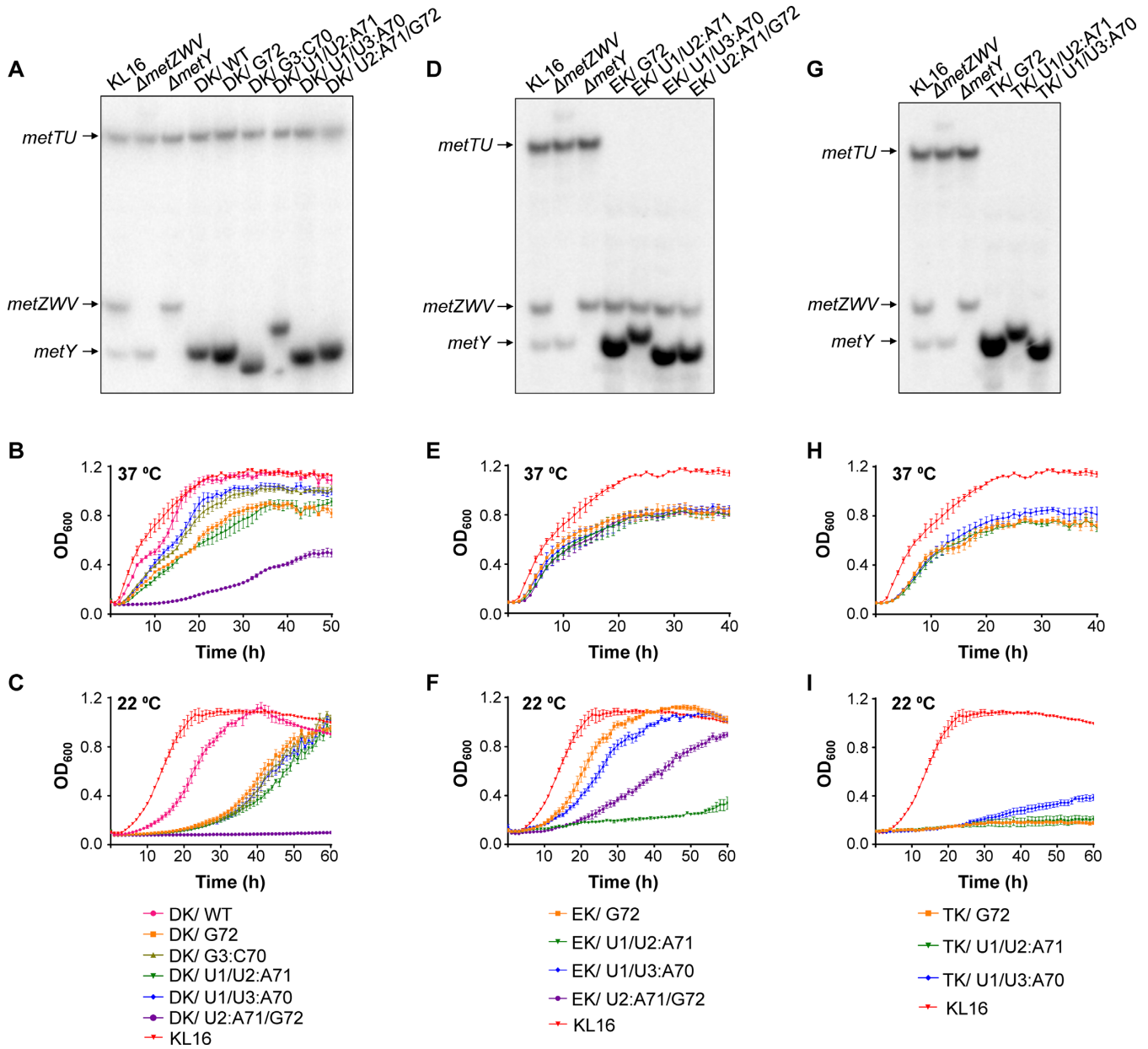


Figure 2. Generation and growth analysis of the knockout strains. (A) Confirmation of the double locus knockout (DK) strains ($\Delta metZWV$ and $\Delta metY$) by northern blot analysis of tRNAs prepared from flask cultures and separated on native PAGE. The tRNA^{Met} (*metT/metU*), tRNA^{MetII} (*metZWV*) and tRNA^{MetIII} (*metY*) are indicated by arrows. Lanes 1, 2 and 3 represent RNA from KL16 (wild type for both the *metZWV* and *metY*) its $\Delta metZWV::cm$ and $\Delta metY$ derivatives, respectively. The plasmid borne tRNA^{Met}, *pmetY** (harboring WT or the mutant tRNA^{Met}, as indicated) were introduced into $\Delta metY$ background and subjected to deletion of the *metZWV* locus (lanes 4–9). (B and C) Growth (in microtiter plates) of the DK strains supported by tRNA^{Met} or its mutants (as indicated) at 37°C and 22°C, respectively. (D) Confirmation of the $\Delta metT$ and $\Delta metU$ strains (EK) by northern blot analysis of tRNAs prepared from flask cultures and separated on native PAGE. Lanes 1, 2, and 3 are as in (A). The plasmids *pmetY** (harboring tRNA^{Met} mutants as indicated) and *pA5* (harboring *leuW-glnU-glnW-glnV-glnX*) were introduced into $\Delta metY$ background and subjected to deletion of the *metT/metU* locus ($\Delta 7::kan$) (lanes 4–7). (E and F) Growth (in microtiter plates) of the tRNA^{Met} gene knockout (EK) strains (as indicated) supported by the mutant tRNAs (EK/*pmetY**) at 37°C and 22°C, respectively. (G) Confirmation of the triple locus knockout ($\Delta metZWV$, $\Delta metY$, $\Delta metT$ and $\Delta metU$ shown as TK) strains by northern blot analysis of tRNAs prepared from flask cultures and separated on native PAGE. Lanes 1, 2, and 3 are as in (A). (H and I) Growth (in microtiter plates) of the TK strains supported by tRNA^{Met} mutants (as indicated) at 37°C and 22°C, respectively. For (B)–(I), error bars indicate standard error of mean (SEM). All growth curve analyses were done at the same time. Data for the parent strain (KL16) have been shown in all panels (B, E, and H at 37°C; and C, F and I at 22°C) for comparison.

TK/U1/U3:A70 on various carbon sources, nitrogen sources and nutrient supplements using the Biolog phenotypic microarray platform. The single knockout strains did not show any appreciable change in growth profile compared to the parent strain (Supplementary Figure S9a and b), but the DK/U1/U3:A70 strain showed mild growth defects on some carbon and nitrogen sources (Supplementary Figure S9c). The triple knockout strains, TK/G72 and TK/U1/U3:A70, showed stronger growth defects on many carbon and nitrogen sources (Supplementary Figure S9d and e), primarily with respect to growth on metabolites which are direct intermediates of the Krebs's cycle, with the defects seen for TK/G72 being larger compared to TK/U1/U3:A70, indicating that the differences observed may be directly related to the growth rate of the strains. Doubling times of the triple knockout strains calculated from growth in LB in flask cultures show that they grow about 1.5–2-fold slower than the vector control (Supplementary Figure S10) with the strains supported by G72, U1/U2:A71, and U1/U3:A70 tRNAs having a doubling time of 61.7, 67.8 and 55 min compared to 35.2 min for the vector control.

Dual-function mutants bind to both IF2 and EFTu *in vivo*

In mitochondrial protein synthesis where the single tRNA^{Met} is partitioned into initiation and elongation steps, the tRNA^{Met} either binds IF2_{mt} or EFTu_{mt} depending on its formylation status. We wondered whether a similar phenomenon operated in the TK strains. To analyze this, we checked for binding of tRNA^{fMet} mutants to N-terminally or C-terminally His₆ appended IF2 or EFTu, respectively following a pulldown with Ni-NTA beads. Expectedly, we detected binding of wild type tRNA^{fMet} (both the fMetI and fMetII) to IF2 in wild type cells (Figure 3B, lane 1). There was negligible binding of tRNA^{fMet} to IF2 (Figure 3B, top panel). The amounts of mutant tRNA^{fMet} bound to IF2 were roughly in the order of their efficiency of initiation as analyzed by the growth of the double knockout (DK) strains supported by the mutants. The binding of these tRNAs to EFTu was also checked. Overexpression of a plasmid encoded EFTu-His gene led to significant binding to even wild type initiator tRNA (Supplementary Figure S11). Thus, a His-tagged copy of EFTu was knocked in into the *tufB* locus using lambda Red mediated recombination (Supplementary Figure S12). Pulldowns performed in this strain background were significantly more specific and indicative of *in vivo* binding. As opposed to wild type tRNA^{fMet} which bound poorly to EFTu, the tRNA^{fMet} mutants capable of supporting elongation function, bound strongly to EFTu (Figure 3E, lane 3, 6 and 7). The only exception was U1/U2:A71, which bound poorly to EFTu (Figure 3E, lane 5). However, it may be noted that this mutant accumulated to low levels in the cell.

Naturally occurring variants of i-tRNAs with a base pair at the 1:72 position

The results shown above provide a basis for the possibility of occurrence of a phenomenon observed in mitochondria, i.e. for the natural occurrence of a single tRNA^{Met} in

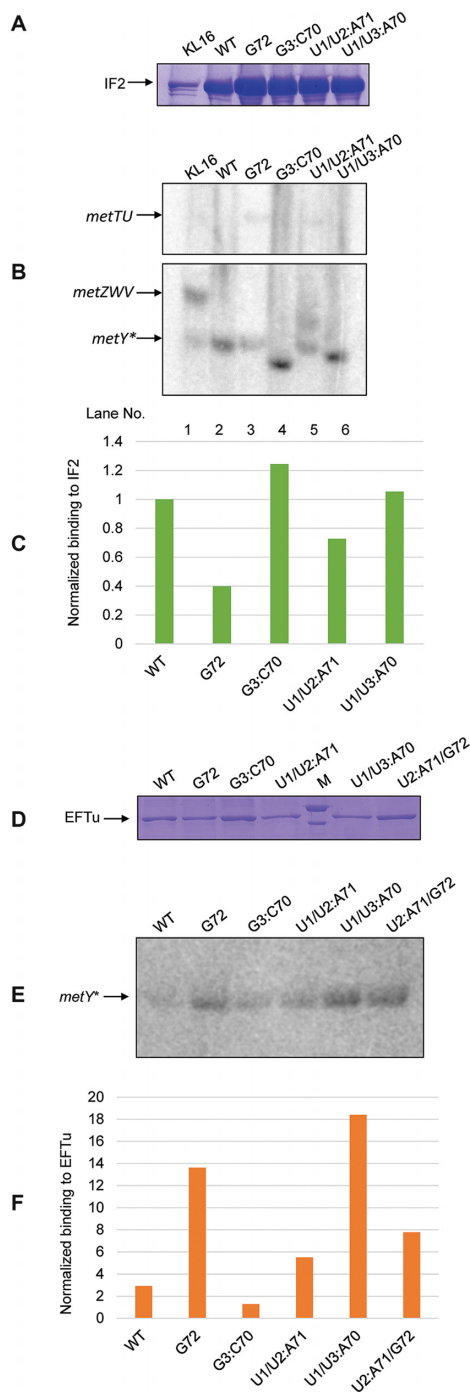


Figure 3. Binding of tRNA^{fMet} or its mutants to IF2 and EFTu. (A) Analysis of IF2 pulldown fractions by 12% SDS-PAGE. Pulldowns were performed in $\Delta metY \Delta metZWV$ (DK) strains supported by the mutant indicated, and harboring a His-tagged plasmid encoded copy of IF2. The band corresponding to full length His-IF2 (~98.5 kDa) is indicated. (B) Northern blot analysis of the pulldown samples separated on 15% native PAGE. IF2 bound RNA fraction was probed with ³²P-5'-end labeled oligomers capable of binding to both fMetI and fMetII tRNAs. Quantification of binding from the northern blot is shown in (C). (D) Analysis of EFTu pulldown fractions by 12% SDS-PAGE. Pulldowns were performed in $\Delta metY tufB$ -His:kan strains harboring the mutant tRNAs indicated. The band corresponding to full length EFTu-His (~44 kDa) is indicated. (E) Northern blot analysis of the EFTu pulldown fractions separated on 15% native PAGE. Northern blot analysis was performed as in (b). Quantification of binding from the northern blot is shown in (F).

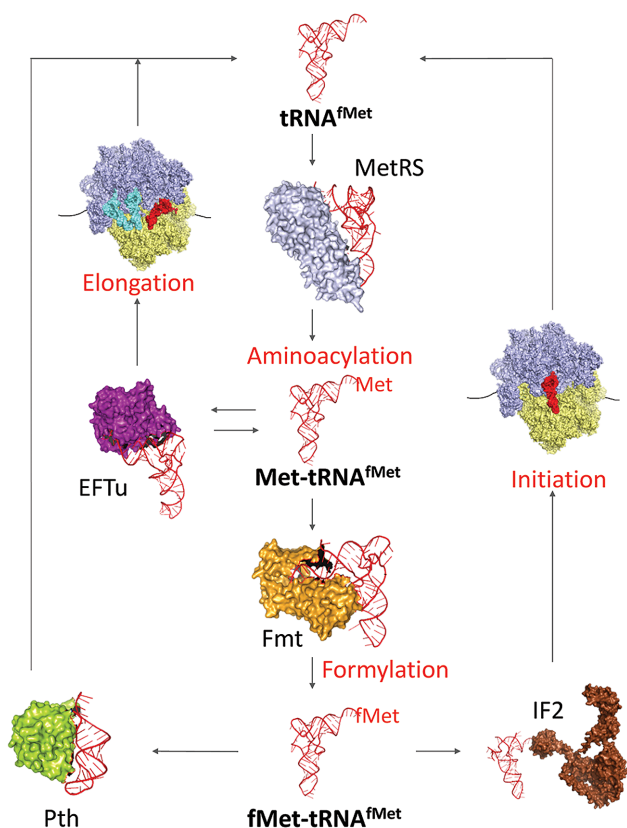


Figure 4. Model for alternate fates of the bacterial initiator tRNA. Following aminoacylation by MetRS, the formylability of the initiator tRNA by Fmt determines its entry into either initiation (by binding of the formylated species to IF2) or elongation (by binding of the unformylated species to EFTu). Partitioning of tRNA^{fMet} between various factors (Fmt/IF2 or EFTu) determines its fate in protein synthesis.

bacterial systems. To test whether variants of i-tRNA which could function in elongation exist naturally, we analyzed the structures of i-tRNAs from all sequenced bacterial genomes to date. We find that there are naturally occurring i-tRNAs which possess the characteristic three consecutive G:C base pairs (or their functional variants) (24) along with a U1:A72 pair (Supplementary Figure S13a–e), U1:G72 wobble pair (Supplementary Figure S13f–k), and even a G1:C72 pair (Supplementary Figure S13l–m) in the acceptor stem. Although many of these organisms do possess separate methionine elongator tRNAs, it may well be that the i-tRNAs with the U1:G72 and G1:C72 base pairs could also carry out elongator tRNA functions, at least under some conditions.

DISCUSSION

Overall, our studies show that by fine tuning formylation efficiencies (by introducing mutations in the acceptor stem of tRNA^{fMet}), it is possible to direct a tRNA into either the initiation or the elongation pathways, providing an insight into the evolution of distinct methionine tRNA species. As Fmt_{mt} primarily recognizes the amino acid attached to the tRNA, mitochondrial tRNA^{Met} could be driven either into the initiation or elongation by regulating the levels of formy-

lase enzyme. As opposed to this, eubacterial Fmt recognizes sequence elements on the tRNA^{fMet} body itself. Although this necessitates the presence of two distinct tRNAs for initiation and elongation functions, we have shown that it is possible to change the fate of the tRNA by altering the formylatability of the tRNA^{fMet}. However, instead of regulation at the level of Fmt expression, shifting the balance of fate in bacterial systems require sequence changes in the determinants of the Fmt enzyme, a mechanism that could have been adopted during the evolution of distinct tRNAs for initiation and elongation. Interestingly, the mutant that is most efficient at sustaining initiation and elongation together, U1/U3:A70, is also the one that is most similar to the human mitochondrial methionine tRNA (Supplementary Figure S1), supporting such an idea.

Mutations in the *MTFMT* gene encoding human mitochondrial formylase cause Leigh syndrome and combined OXPHOS deficiency (25). Such patients show a significant reduction in the levels of fMet-tRNA^{Met}, with a severe defect in mitochondrial translation. Interestingly, the triple knockout strains supported by the U1/U2:A71 mutant (which has the least amount of formylated species at steady state, Figure 1E) shows slowest growth among the three triple knockout strains in our *E. coli* knockout system. In addition, our study provides insights into bacterial protein synthesis and the delicate balance of protein factors and sequence elements in the acceptor stem of tRNA^{fMet} that decide the fate of the bacterial i-tRNA (Figure 4).

We noted the existence of i-tRNA variants that harbor U1:A72 pair, U1:G72 wobble pair, or a G1:C72 pair in the acceptor stem in many of the sequenced bacterial genomes. Although not known so far, our observation provides a basis for the possibility of natural occurrence of a single tRNA system for initiation and elongation in at least some bacteria. Importantly, together with our earlier studies (26,27), our current finding reinforces the use of *E. coli* as a model to investigate the evolutionary and mechanistic aspects of the mitochondrial protein synthesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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