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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 (*metZWV*), 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 *metZWV* and

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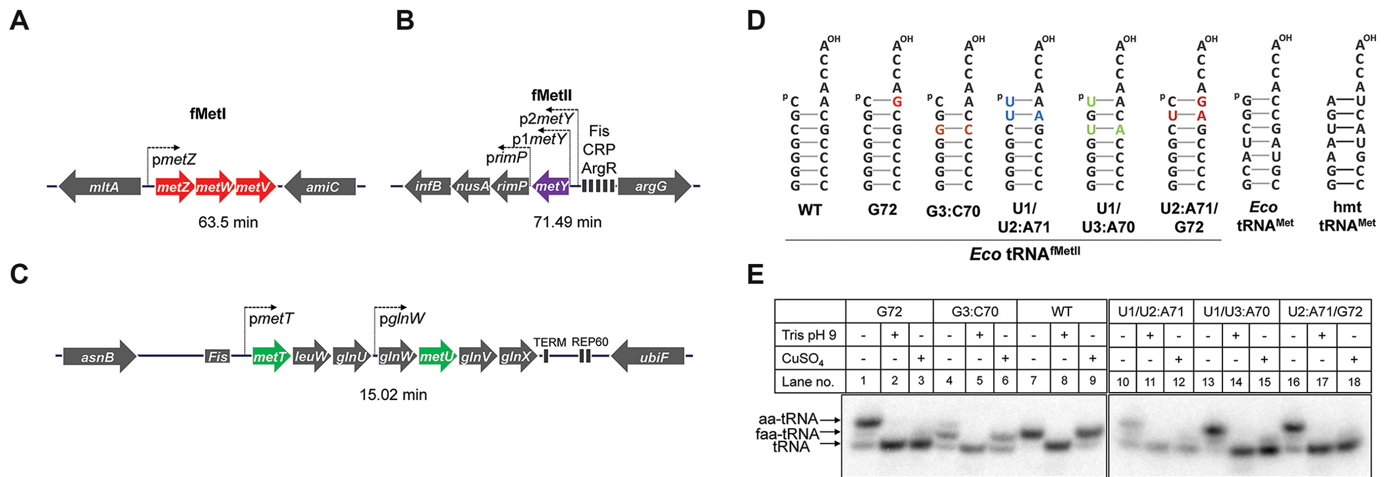

Figure 1. Mutants of the *E. coli* initiator tRNA and their *in vivo* status. Schematic of organization of the initiator (**A** and **B**) and elongator (**C**) methionine tRNA gene loci in *E. coli.* fMetI encoding tRNA genes (*metZWV*) are shown in red, the fMetII encoding gene (*metY*) is shown in 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 ^{32}P-5′-end labeled oligomers complementary to nucleotides 39–55 of *metY*. Total tRNA was isolated from *ΔmetY* 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 $tRNA^{fMet}$ (*metZWV* 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*_NcoIfp and *glnX*_HindIIIrp. 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*_NcoIfp, *glnW*_EcoRIrp, *glnV*_EcoRIfp and *glnX*_HindIIIrp 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_Hisfp, IF2_rp, EFTu_fp and EFTu_Hisrp 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

ΔmetZWV::cm. *E. coli* KL16/pKD46 was grown at 30°C to 0.2 OD_{600}, induced with 1 mM arabinose and made electrocompetent at 0.7 OD_{600} by three successive washes in sterile 10% glycerol, obtaining a 100-fold concentration of cells in the process. A 50 μl 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 *metZWV* locus using the primers *metZWV*_KOfp and *metZWV*_KOrp. About 300 ng of the 1 kb amplicon was electroporated into the cells at 1.8 kV, 200 Ω and 25 μF 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 *metZWV*_fp and *metZWV*_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 *metZWV* locus (Supplementary Figure S2).

Δ*metY*. An *E. coli* KL16 strain with the *metY* gene replaced by a chloramphenicol cassette, *ΔmetY::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 *Δ7*::*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*_KOfp2 and *metT*_KOrp2. 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 (*ΔmetZWV*::*cm ΔmetY*/p*metY**; also referred to as DK/p*metY**) were generated by P1 phage transduction mediated transfer of the *ΔmetZWV::cm* locus from *E. coli* KL16 *ΔmetZWV*::*cm* to *ΔmetY*/p*metY**. Strains were selected on LB agar containing 30 μg/ml chloramphenicol and 7.5 μg/ml tetracycline. Transductants were verified by colony PCR using *metZWV*_fp and *metZWV*_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 *metZWV* 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} (*Δ7*::*kan ΔmetY::cm*/p*metY**/pA5; renamed as EK/p*metY**) were generated by P1 transduction mediated transfer of the *Δ7::kan* locus from *Δ7::kan*/pT6 strain to *ΔmetY*::*cm*/pA5/p*metY** 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 (*ΔmetZWV*::*cm ΔmetY Δ7*::*kan*/p*metY**/pA5; referred to as TK/p*metY**) were generated using the *ΔmetZWV*::*cm ΔmetY* strain constructed as above. The double knockout strains harboring pA5 were transduced with P1 phage raised on *Δ7*::*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.

ΔmetY 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 *ΔmetY* by P1 mediated transduction to obtain KL16 *ΔmetY 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 OD_{600} 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 OD_{600} was measured. The linear part of the curve was identified by plotting the natural log of OD_{600} 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 OD_2 and OD_1 are the OD_{600} values at times t_2 and t_1.

IF2 and EFTu pulldowns

IF2 and EFTu pulldowns were performed in DK/p*metY**/pIF2 and either *E. coli* KL16/p*metY**/pEFTu or KL16 *ΔmetY tufB*-His*:kan*, respectively. About 300 ml of each culture was grown to about 1.0 OD_{600} 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 $CaCl_2$ 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-equilibriated 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 ^{32}P 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 ($tRNA^{Met}$) 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 *ΔmetZWV::cm* donor strain (deleted for the *metZWV* locus) was used to transduce the *ΔmetY* recipient strains harboring plasmid borne genes for the wild type or mutant tRNA^{fMet} (indicated as p*metY**). The resulting transductants (*ΔmetZWV::cm ΔmetY*/p*metY**) referred to as double locus knockout strains (DK/p*metY**) were deleted for all four tRNA^{fMet} genes (*metZWV* 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/p*metY*) 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 *ΔmetZWV* 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 suboptimal 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 p*metY** (tRNA^{fMet} harboring plasmids). We then followed a two-step protocol. In the first step, we generated *Δ(metT-leuW-glnU-glnW-metU-glnV-glnX)::kan*/pT6 strain (referred to as *Δ7::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 *ΔmetY::cm*/pA5/p*metY** strain (recipient) with the P1 raised on *Δ7::kan*/pT6 (donor) strain (Supplementary Figure S7b). The resulting transductants (*ΔmetY::cm Δ7::kan*/pA5/p*metY**; referred to as elongator knockout strains, EK/p*metY**) 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/p*metY** 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/p*metY** 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/p*metY** strains are deleted for *metY*, they possess the *metZWV* 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 *metZWV* locus from the EK/p*metY** 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/p*metY**) supported by any of the four tRNA^{fMet} mutants with the P1 raised on the *Δ7::kan* strain to obtain triple locus knockout (TK) strains deleted for all copies of the chromosomal tRNA^{fMet} and tRNA^{Met} genes (*ΔmetY ΔmetZWV::cm Δ7::kan*/pA5/p*metY** (or TK/p*metY**) (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), *ΔmetY*, *ΔmetZWV::cm*, DK/U1/U3:A70, TK/G72 and

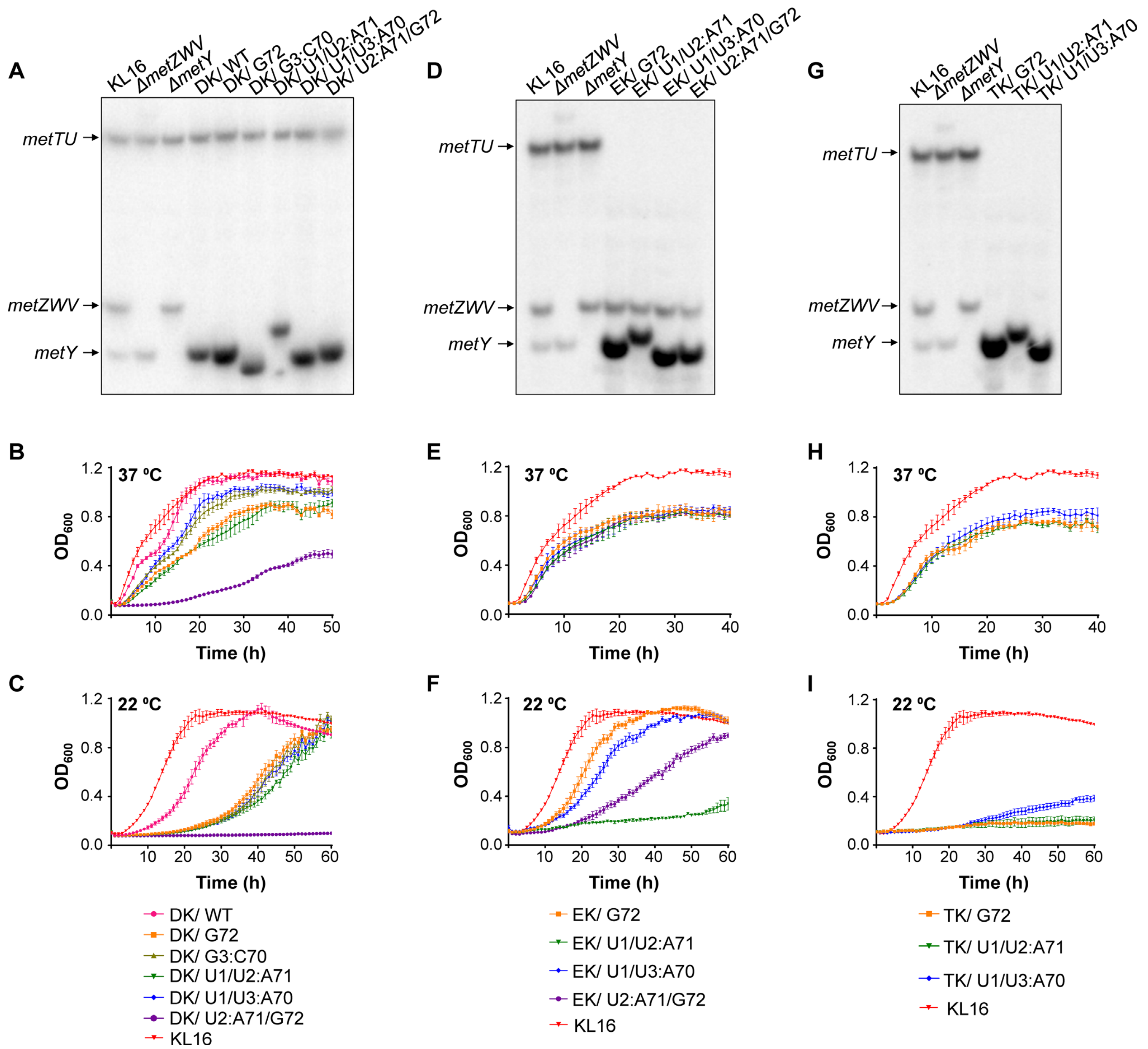

Figure 2. Generation and growth analysis of the knockout strains. (**A**) Confirmation of the double locus knockout (DK) strains (*ΔmetZWV* and *ΔmetY*) by northern blot analysis of tRNAs prepared from flask cultures and separated on native PAGE. The $tRNA^{Met}$ (*metT*/*metU*), $tRNA^{fMetI}$ (*metZWV*) and $tRNA^{fMetII}$ (*metY*) are indicated by arrows. Lanes 1, 2 and 3 represent RNA from KL16 (wild type for both the *metZWV* and *metY*) its *ΔmetZWV*::*cm* and *ΔmetY* derivatives, respectively. The plasmid borne $tRNA^{fMet}$, p*metY** (harboring WT or the mutant $tRNA^{fMet}$, as indicated) were introduced into *ΔmetY* 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 $tRNA^{fMet}$ or its mutants (as indicated) at 37°C and 22°C, respectively. (**D**) Confirmation of the *ΔmetT* and *ΔmetU* 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 p*metY** (harboring $tRNA^{fMet}$ mutants as indicated) and pA5 (harboring *leuW-glnU-glnW-glnV-glnX*) were introduced into *ΔmetY* background and subjected to deletion of the *metT*/*metU* locus (*Δ7*::*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/p*metY**) at 37°C and 22°C, respectively. (**G**) Confirmation of the triple locus knockout (*ΔmetZWV*, *ΔmetY*, *ΔmetT* and *ΔmetU* 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 $tRNA^{fMet}$ 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 Kreb'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 His_6 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 $tRNA^{Met}$ 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 knockedin 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 occuring 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

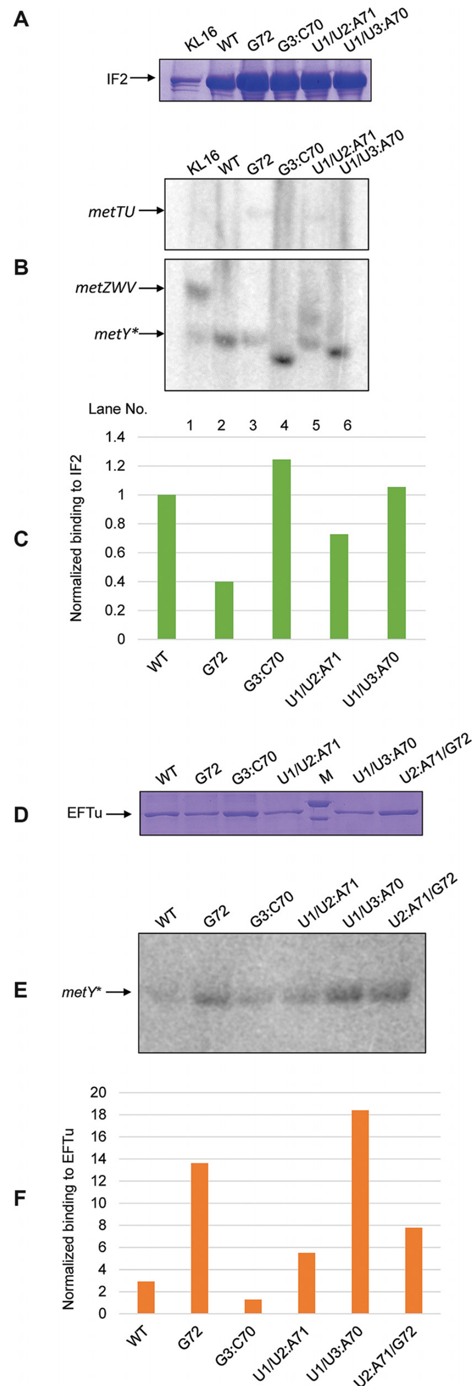

Figure 3. Binding of $tRNA^{fMet}$ or its mutants to IF2 and EFTu. (**A**) Analysis of IF2 pulldown fractions by 12% SDS-PAGE. Pulldowns were performed in *ΔmetY ΔmetZWV* (DK) strains supported by the mutant indicated, and harboring a His-tagged plasmid encoded copy of IF2. The band corresponding to 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 ^{32}P-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 *ΔmetY 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**).

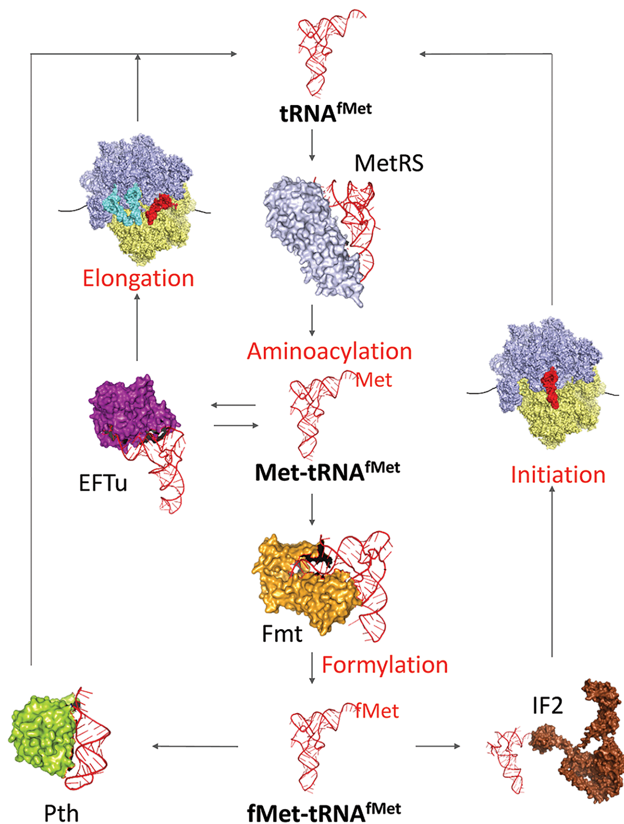

Figure 4. Model for alternate fates of the bacterial initiator tRNA. Following aminoacylation by MetRS, the formylability of the initiator tRNA by Fmt determines its entry into either initiation (by binding of the formylated species to IF2) or elongation (by binding of the unformylated species to EFTu). 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 formylase 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 $fMet\text{-}tRNA^{Met}$, 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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