Role of tRNA Orthogonality in an Expanded Genetic Code

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ABSTRACT: We found that Methanococcus jannaschii DSM2661 tyrosyl-tRNA synthetase (Mj E9RS), specifically evolved to charge its cognate tRNA with the unnatural amino acid p-acyethylphenylalanine (pAcF) in E. coli, misaminoacylates the endogenous E. coli prolyl-tRNAs with pAcF at a low level (0.5% per proline frequency) in both the absence or presence of its co-evolved amber suppressor tRNA (M. jannaschii tyrosyl-tRNA, tRNA_{Mj}^{AcF,Tyr}). In contrast to other E. coli tRNAs, the identity elements for recognition of the prolyl tRNAs by the E. coli prolyl-tRNA synthetase (C1, G72, and A73) are similar to those in tRNA_{AcF,Tyr}^{E.coli}. Although the unique acceptor stem identity elements of the prolyl-tRNAs likely lower their recognition by the other endogenous aaRSs in E. coli, resulting in enhanced fidelity in the wild type strain, they lead to misaminoacylation by the archaea-derived E9RS. Misincorporation of pAcF for proline was resolved to below detectable levels by overexpression of the endogenous E. coli prolyl-tRNA synthetase (proS) gene in combination with additional genomic manipulations to further increase the intracellular ratio of the ProS over its cognate proline tRNAs. These experiments suggest another mechanism by which the cell maintains the high fidelity of protein biosynthesis.
poly(ethylene glycol) (PEG) polymer. rhFGF-21-pAcF produced in high density bacterial fermentation (2.5 g per liter) using a pAcF specific trNA_{CUA}/aaRS pair afforded other higher molecular weight PEGylated species in addition to the expected mono-PEGylated protein. The earliest processing stage in which the high molecular weight PEGylated species were observed was also investigated, and it was determined that unpurified protein from solubilized inclusion bodies, when subjected to PEGylation, also yielded multiple PEGylated products. Possible causes of the higher molecular weight protein species in the PEGylation reaction were systematically addressed and ruled out, including protein—protein aggregation, protein oligomerization via disulfide bond formation, and high molecular weight contaminants in the PEG raw material reagent. Subsequent reduced intact mass analysis of the mutant protein revealed a ~17% containment of a +92 Da species (Figure 2).

It was observed by peptide mapping of analyte enriched for the +92 Da species by RP -HPLC that the modification was present across multiple peptides and not limited to a specific site (Figure 3). Mass spectral analysis of the tryptic digest revealed that this contaminant corresponded to substitution of proline with pAcF at various sites (Table 1). Because rhFGF-21 has 22 endogenous prolines, misincorporation of pAcF for Pro at a 0.5% frequency afforded a clearly detectable impurity. To confirm this observation, wild-type (wt) rhFGF-21 was produced in the presence of the orthogonal tRNA_{CUA}/E9RS without any amber mutant in the rhFGF-21 gene, and the +92 Da species was still observed at roughly the same abundance. There was, however, a significant difference in relative misincorporation depending on the type of media that was used and the temperature of induction for rhFGF-21 expression, which could be due to previously reported effects.
of bacterial growth rates, relative amino acid and tRNA abundances and codon usage.

Misincorporation due to misrecognition at the level of codon–anticodon interactions is unlikely as there is no real bias of pAcF at different proline sites encoded by the canonical codons (Figure 4), and base pairing, including the wobble rule, does not allow the amber suppressing anticodon CUA to recognize any of the 4 proline codons (CCU, CCC, CCA, CCG). It is possible that the E. coli prolyl-tRNA synthetase aminoacylates these tRNAs with pAcF. Indeed, deletion of the Mj aaRS gene from a plasmid encoding wt rhFGF-21 and tRNA_CUA (plasmid AXID2103) eliminated misincorporation, whereas deletion of the suppressor tRNA_CUA (plasmid AXID2083) had no detectable effect on misincorporation (Figure 5). The bacterial tRNAPro family is the only set of tRNAs to have a C1:G72 base pair in their acceptor stem, which is the same identity element used by archaea (and distinct from the other E. coli tRNAs). In fact a single substitution at G72 in tRNAPro leads to a large decrease in aminoacylation by the endogenous prolyl-tRNA synthetase.21–23

An alternative explanation for misincorporation of pAcF at proline sites is that the M. jannaschii pAcF specific aaRS to some degree recognizes the E. coli proline tRNA family and aminoacylates these tRNAs with pAcF. Indeed, deletion of the Mj aaRS gene from a plasmid encoding wt rhFGF-21 and tRNA_CUA (plasmid AXID2103) eliminated misincorporation, whereas deletion of the suppressor tRNA_CUA (plasmid AXID2083) had no detectable effect on misincorporation (Figure 5). The bacterial tRNAPro family is the only set of tRNAs to have a C1:G72 base pair in their acceptor stem, which is the same identity element used by archaea (and distinct from the other E. coli tRNAs). In fact a single substitution at G72 in tRNAPro leads to a large decrease in aminoacylation by the endogenous prolyl-tRNA synthetase.21–23 In addition the nucleotides between A14 and U18 are conserved between the E. coli tRNAPro family and the Mj tRNA_CUA as is the short variable loop.

Table 1. Theoretical Tryptic Digest of rhFGF-21-pAcF

| peak no. | from—to | tryptic sequence |
|----------|---------|-----------------|
| T1       | 1–18    | MHPIDSSPLQQFGGQVR |
| T2       | 19–20   | QR               |
| T3       | 21–37   | YLYTDQAQTEAHLEIR |
| T4       | 38–57   | EDGTVGGAADQPSLILQLK |
| T5       | 58–70   | ALKPGVQILGLVK   |
| T6       | 71–73   | TSR              |
| T7       | 74–97   | FLCQRDPGALYSLHFDEACSFR |
| T8       | 98–123  | ELLLEDGNYVGSEQHGLPLHFGNK |
| T9       | 124–127 | SPhR             |
| T10      | 128–132 | DPAPR            |
| T11      | 133–136 | GPAR             |
| T12      | 137–176 | FLPGLPAPPEPGILAPQPDPVGSDDPLSMVGPQGR |
| T13      | 177–182 | SPYAS            |

Prolines are highlighted where the +92 Da species was observed by peptide mapping. The peak numbers T1–T13 correspond to the peptide mapping analysis in Figure 3.
If misrecognition of tRNA\textsuperscript{Pro} by the pAcF aaRS is the cause of misincorporation, overexpression of the \textit{E. coli} prolyl-tRNA synthetase gene (\textit{proS}) should reduce or eliminate this phenomenon by competing with the \textit{Mj} pAcF RS for the acylation of endogenous tRNA\textsuperscript{Pro} with proline rather than pAcF. To this end we subcloned the \textit{E. coli} K-12 W3110 proS gene, which has a temperature-sensitive (≥34 °C) tryptophan to arginine mutation at residue 375, behind its own promoter in the plasmid AXID2191, and transformed the cell line W3110B57 with this expression plasmid.\textsuperscript{19} At 30 °C there was no pAcF misincorporation detected due to increased expression of the mutant proS gene (Figure 6), nor was there any significant effect on mutant rhFGF21 expression levels. At 37 °C pAcF misincorporation was restored at proline residues in rhFGF-21am as the mutant ProS is not functional at this lethal temperature, i.e., only the wild-type protein is functional and translated from the transcript of the genomic copy of \textit{proS}. Thus it appears that misincorporation of pAcF for proline in these experiments is due to a low level of misrecognition of tRNA\textsuperscript{Pro} by the pAcF aaRS.

Why is misincorporation of the UAA observed only with proline? Proline is the only amino acid with a secondary amine, and as a consequence its incorporation into the growing polypeptide chain is 3–4 orders of magnitude slower than the incorporation of the other canonical amino acids.\textsuperscript{24–26} Thus if the endogenous \textit{E. coli} ProS misaminoacylates tRNA\textsuperscript{Pro} with any other canonical amino acid, that amino acid will be incorporated into peptide at the A site (assuming little EF-Tu bias) more rapidly than proline.\textsuperscript{27} Therefore since binding to the A site by the tRNA-EF-Tu complex is reversible,\textsuperscript{22,27} there will be an inherent bias for misincorporation by misacylated tRNA\textsuperscript{Pro} relative to other misacylated tRNAs. ProS has an active site that can broadly accept a range of small hydrophobic amino acids but contains an editing domain to ensure genetic code integrity. However, misincorporation can also occur by binding and aminoacylation of any of the tRNA\textsuperscript{Pro} by non-proline specific aaRSs. By mutation of the tRNA identity element from G1:C72 to the archaeal C1:G72 base pair, the \textit{E. coli} tRNA\textsuperscript{Pro} increases its orthogonality to the other bacterial tRNA/aaRS pairs and further decreases the potential for misincorporation of another canonical amino acid at proline sites. This strategy, in addition to editing, EFTu binding, and post-aminoacylation enzymatic processing, likely represents yet another mechanism by which \textit{E. coli} can ensure high fidelity in mRNA translation. However, it is also clear that this mechanism is not used universally, so other mechanisms likely exist to ensure high fidelity. Unfortunately, the C1:G72 base pair makes the bacterial tRNA\textsuperscript{Pro} similar to our archaeal \textit{Mj} orthogonal tRNA\textsubscript{Clu}/aaRS pair and leads to misincorporation. This misincorporation is overcome by overexpressing of the proS gene in the host strain.

The cross-talk among the cellular components of the translation machinery (the last step in the Central Dogma) is widely accepted to have been evolutionary balanced so that it is not detrimental to the proteome or the cell itself. One of the results of this balancing act is the intrinsic intracellular ratio of the matured, fully processed tRNAs to their cognate synthetases (which almost certainly changes as cells go through various physiological states). In our synthetic strains with a 21 amino acid code, due to the overlapping recognition elements in both \textit{E. coli} proline and \textit{M. jannaschii} tyrosine tRNAs, \textit{M. jannaschii} tyrosyl-tRNA synthetase aminoacylates available uncharged endogenous proline tRNAs. When the \textit{E. coli} endogenous proS is overexpressed in the cell by increasing its copy number, the phenotype becomes undetectable by LC–MS/MS since the balance shifts toward the endogenous ProS and the remaining excess, if any, of the free proline tRNAs becomes insufficient for the misaminoacylation. Thus, in this case one consequence of adding additional translational components, i.e., an orthogonal tRNA/aaRS pair, is that the levels of the endogenous prolyl-tRNAs must be altered to ensure high fidelity in protein biosynthesis, underscoring the degree to which the protein translational machinery has been evolutionary optimized.

![Figure 6](image)

**Figure 6.** LC–MS analyses of rhFGF-21-pAcF from the optimized expression plasmid, AXID2292 at (A) 30 °C and (B) 37 °C. The expected mass of intact rhFGF-21 is 19587.1 Da; the observed mass is 19587.4 Da for both constructs. Peaks labeled as “Δ” are Δ92 Da species. Map symbols: P\textsubscript{acF}; \textit{E. coli} arabinose operon dual regulator gene; P\textsubscript{pro}A; arabinose operon promoter; Ec \textit{proS}; \textit{E. coli} wild type prolyl-tRNA synthetase gene.

#### METHODS

**E. coli Cell Line Construction.** The wild-type \textit{E. coli} K-12 W3110 cell line with GenBank accession no. AP009048.1 was purchased from ATCC, Virginia (catalog no. 2732S). Through homologous recombination the T7 RNA polymerase gene cassette was inserted into the W3110 genome.\textsuperscript{18} In this newly created cell line the \textit{ftsu} gene was replaced with \textit{difS} (Trimethoprim resistance marker) to create the W3110B55 cell line. In W3110B55 the \textit{ompC} gene was replaced with the chloramphenicol resistance marker (\textit{cat}) to create the W3110B57 cell line. In a similar manner the \textit{proS} W375SR:cat (point mutation for substitution of tryptophan with arginine) was generated to create a temperature-sensitive (ts) cell line W3110B60.\textsuperscript{17} The W375SR point mutation in \textit{proS} conferred a lethal host phenotype at temperatures ≥34 °C.

**Construction of Expression Plasmids.** The expression plasmids were constructed using pET-20b (+) and pEF-24 (+) plasmids (EMD Biosciences, California). The amber codon suppression cassette consisting of the modified \textit{M. jannaschii} tyrosyl tRNA synthetase was inserted at the BamHI site. The rhFGF-21am gene of interest (also known as AXP-000-028am) with the amber codon (TAG) within the ORF at glutamine position 108 was inserted downstream of the bacteriophage T7 promoter at the NdeI and KpnI restriction sites in this plasmid. The wild-type \textit{E. coli} K-12 W3110 proS gene was inserted into the vector at the BglII restriction site. The temperature sensitive phenotype of the W3110B60 was complemented by the wild-type copy of the proS gene in the expression plasmid, AXID1395 corresponds to the amber expression plasmid with the Ambrr suppression elements (\textit{Mj} tyrosine tRNA\textsuperscript{MjTyr} and \textit{Mj} tyrosine tRNA synthetase, E9RS) and rhFGF-21-Q108am (AXP-000–028am). The AXID2089 plasmid has the rhFGF-21-Q108am gene but lacks the
amber suppression elements. The AXID2083 plasmid contains the rhFGF-21-Q108am gene and the Mj tyrosine tRNA_{ACU}_{Mj} but lacks the Mj E9RS synthetase. The AXID2103 plasmid includes only the rhFGF-21-Q108am gene and the Mj E9RS synthetase. The AXID2292 plasmid encodes the rhFGF-21-Q108am, Mj tyrosine tRNA_{ACU}_{Mj}, Mj E9RS synthetase, and the E. coli K-12 W3110 proline synthetase (pAcF) under the arabinose promoter.

**Production of pAcF Containing rhFGF-21.** Chemically competent DH5α-T1 cells (Life Technologies, California) were transformed with the expression plasmid containing rhFGF-21am, the system components for pAcF incorporation, and the proS gene. Plasmid DNA from a single colony was extracted and used for transformation of chemically competent E. coli K-12 W3110B60 cells.14 rhFGF-21-pAcF protein production was confirmed through SDS-PAGE and LC–MS analyses of a 500 mL shake flask experiment using defined medium (minimal medium supplemented with leucine, isoleucine, trace metals, and vitamins) with 50 μg/mL of kanamycin sulfate (Sigma, Missouri) at 37 °C. This result was further validated and confirmed through similar methods in high cell density fermentations consisting of batch and fed-batch phases.

**High Cell Density Fermentations.** The fermentation process for production of rhFGF-21-pAcF consists of two stages: (1) inoculum preparation and (2) fermentor production of rhFGF-21-pAcF. The inoculum is started from a single vial, thawed, diluted 1:1000 (v/v) into 50 mL of defined seed medium in a 250 mL baffled Erlenmeyer flask, and incubated at 37 °C and 250 rpm. The fermentation is batched with 4 L of chemically defined medium that utilizes glycerol as a carbon source. The seed culture is added to fermentor to an initial OD_{600nm} of 0.05. Dissolved oxygen is maintained at 30% air saturation by face flow rate of 0.15 L/min. The temperature and pH are controlled at 37 °C and 7.0, respectively. When the culture reaches an OD_{600nm} of 35 ± 5, feeding commences at a rate of 0.25 mL/L/min. Consequently, L-Ala-pAcF dipeptide is added at 0.4 g/L. Fifteen minutes after the addition of dipeptide, the culture is induced with L-arabinose at a final concentration of 2 g/L. The culture is harvested at 6 h post induction.

**Inclusion Body Preparation.** Cell paste (150–250 μg) was resuspended with 1 mL of Bugbuster (EMD4 Biosciences, California) and 2 μL of Lysonase (EMD4 Biosciences, California) and incubated at 25 °C with shaking (Thermomixer) at 1400 rpm for 15 min. The sample was centrifuged at 16000 × g for 15 min, and the supernatant was removed by aspiration. The Bugbuster, Lysonase treatment and supernatant removal was repeated twice. The inclusion body was solubilized with 500 μL of 20 mM Tris, pH 8.0, 8 M Guanidine-HCl, and 0.1 M diithiothreitol (DTT). The sample was incubated at 45 °C with shaking at 1400 rpm for 30 min. Approximately 100 μL of the solubilized inclusion body was buffer exchanged into 1× PBS pH 7.4 using a Zeba spin column (Pierce, Illinois). The sample was diluted 1:1 with solubilization buffer and then applied to a 0.22 μm spin filter (Millipore, Massachusetts).

**HPLC and Mass Spectrometry.** Samples were applied onto a POROS R2 10 μm column, 21 mm × 30 mm (Applied Biosystems, California) using an Agilent 1200 HPLC in tandem with an Agilent 6510 Q-TOF. The column was equilibrated in 80% mobile phase A (0.05% TFA in 98% HPLC H₂O/2% acetonitrile) and 20% mobile phase B (0.04% TFA in 90% acetonitrile and 10% HPLC H₂O) with an increase to 65% mobile phase B over 15 min with a flow rate of 0.15 mL/min. The acquired spectra were deconvoluted using Agilent’s Bioconfirm software (Agilent Technologies, California). The TIC was also integrated with Chemstation settings for quantitation.

**Peptide Mapping.** The rhFGF-21-pAcF protein was reduced in 6 M guanidine-HCl, 0.1 M Tris, pH 8.0, 0.05 M DTT for 1 h at 37 °C. The reduced samples were alkylated with 0.1 M iodoacetamide at RT in the dark for 40 min followed by quenching with 0.1 M DTT. Samples were buffer exchanged into 50 mM Tris, 5 mM calcium chloride, pH 7.5 followed by trypsin addition at 1:20 (trypsin:protein) and incubated for 18 h at 37 °C. Peptide mapping samples were loaded onto an Agilent SB-C18 2.1 mm × 150 mm column with 100% mobile phase A (0.05% TFA in 98% HPLC H₂O/2% acetonitrile) and eluted with a gradient of 0.5%/min with mobile phase B (0.04% TFA in acetonitrile) over 68 min. The flow rate was 0.2 mL/min, and column temperature was set to 50 °C.

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