In vivo formation of glutamyl-tRNA^{Gln} in *Escherichia coli* by heterologous glutamyl-tRNA synthetases

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Abstract **Two types of glutamyl-tRNA synthetase exist: the discriminating enzyme (D-GluRS) forms only Glu-tRNA^{Glu}, while the non-discriminating one (ND-GluRS) also synthesizes Glu-tRNA^{Gln}, a required intermediate in protein synthesis in many organisms (but not in *Escherichia coli*). Testing the capacity to complement a thermosensitive *E. coli gltX* mutant and to suppress an *E. coli trpA49* missense mutant we examined the properties of heterologous *gltX* genes. We demonstrate that while *Acidithiobacillus ferrooxidans* GluRS1 and *Bacillus subtilis* Q373R GluRS form Glu-tRNA^{Glu}, *A. ferrooxidans* and *Helicobacter pylori* GluRS2 form Glu-tRNA^{Gln} in *E. coli* in vivo.**

Key words: Misacylated tRNA; tRNA specificity; Glutamyl-tRNA synthetase; Glutaminyl-tRNA synthetase

1. Introduction

Faithful protein biosynthesis relies on the specific aminoacylation of cognate tRNA with the corresponding amino acid by aminoacyl-tRNA synthetases. While many organisms contain one synthetase for each of the 20 canonical amino acids, the mechanism of Gln-tRNA biosynthesis differs in the three domains of life [1]. Glutaminyl-tRNA synthetase (GlnRS) forms Gln-tRNA in eukaryotes and certain bacteria. However, all known archaea, most bacteria and some eukaryotic organelles lack GlnRS [1,2]. In these cases Gln-tRNA is generated in a two-step pathway. A non-discriminating glutamyl-tRNA synthetase forms Glu-tRNA^{Gln} which is converted to the cognate Gln-tRNA^{Gln} by a tRNA-dependent Glu-tRNA^{Gln} amidotransferase (Glu-AdT). *Bacillus subtilis* is the best-studied example among organisms utilizing the transamidation pathway for Gln-tRNA^{Gln} synthesis. A single ND-GluRS, a heterotrimeric Glu-AdT, and the absence of a gene encoding GlnRS characterize *B. subtilis* in this regard [3,4].

Analysis of bacterial genomes reveals that *Escherichia coli* (with a discriminating GluRS and a GlnRS) and *B. subtilis* (with a single ND-GluRS and Glu-AdT) possess the 'prototypes' of the machinery forming Glu-tRNA and Gln-tRNA. However, duplicated GluRS enzymes are found in many organisms including *Acidithiobacillus ferrooxidans* and *Helicobacter pylori* [5,6]. To shed further light on the nature of GluRS enzymes, we set out to develop an *E. coli* in vivo system for this purpose. Based on the availability of characterized missense suppression systems [7], and on the capability of *E. coli* to tolerate in vivo mischarged Asp-tRNA^{Asn} [8], we reasoned that Glu-tRNA^{Gln} may also be tolerated. We studied (i) a tryptophan synthetase-based *E. coli* genetic system [9] and (ii) a thermosensitive *E. coli gltX* mutant [10] to assess the formation of Glu-tRNA^{Gln} and Glu-tRNA^{Glu} respectively by transformation with heterologous *gltX* genes from *A. ferrooxidans*, *H. pylori* and *B. subtilis*.

2. Materials and methods

2.1. Plasmids and strains

GluRS1 and GluRS2 genes from *A. ferrooxidans* ATCC23270 as well as the *H. pylori* 26695 GluRS2 gene were polymerase chain reaction (PCR) amplified and cloned into the *Eco*RI site of the pGEX2t (Amersham Pharmacia Biotech) expression vector. *B. subtilis* Q373R and *E. coli gltX* were cloned in the *Nde*I/*Bgl*II sites of pCBS1 [8]. *E. coli trpA* wild-type and *trpA49* variants were cloned in the *Pst*I/*Bam*HI restriction sites of the pMLC210 vector [11] after PCR amplification, generating the corresponding pMLC*trpA49* variants. *TrpA49* carrying either the CAG or CAA glutamine codon at position 49 and *B. subtilis gltX* with the Q373R replacement were constructed by PCR mutagenesis [12] using the following mutagenic primers: 5′-CTGACGCGCTGCAGTTAGGTAC, 5′-GATACCTAACTGCAGCGCGTCAG, 5′-CTGACGCGCTGCAATTAGGTAC, 5′-GATACCTAATTGCAGCGCGTCG, 5′-GTATCATGAGCGATTAAGCTACGG, and 5′-CCGATGCTTAATCGCTCATGATACG (underlined are indicated the mutated codons). Because of the different origin of replication, plasmids derived from pGEX2t and pCBS1 are compatible with pMLC210 derivatives. Plasmids were specifically selected by resistance to ampicillin or chloramphenicol respectively. *E. coli* DH5α or JM109 were used for cloning experiments. *E. coli* JP1449 [10] carrying a thermosensitive mutation in *gltX* (encoding the GluRS) was used in complementation assays with heterologous *gltX*. *E. coli* strain KS463 (*E. coli* Genetic Stock Center #5644) carrying a *trpA33* [13] inactivating mutation was used as recipient in the missense suppression tests. *E. coli* A2/A2 was used for the synthesis of indole 3-glycerol phosphate (IGP), the substrate for tryptophan synthetase assay [14]. All DNA constructs and mutagenesis were confirmed by DNA sequencing.

2.2. Restoration of the thermostable phenotype of E. coli gltX strain

E. coli JP1449 was transformed with each of the plasmids containing heterologous *gltX* genes from the sources described above. Ampicillin-resistant colonies obtained at 30°C on Luria–Bertani (LB) agar plates were streaked on a new plate and incubated at 42°C. Plates were scored every 12 h.

2.3. Suppression of E. coli trpA49 missense mutations

E. coli strain KS463 carries the inactivating mutation *trpA33* [13] which results in tryptophan auxotrophy. Cells were co-transformed by electroporation with one of the variants of plasmid pMLC*trpA49*

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carrying either glutamate (GAA) or glutamine (CAA or CAG) codons at position 49, and plasmids carrying heterologous *gltX* genes. Transformants were plated on LB agar with both ampicillin and chloramphenicol and incubated for 16 h at 37°C. Colonies were transferred to 3 ml of LB liquid medium in the presence of antibiotics and cultured for 16 h at 37°C. Subsequently, cells were washed with M9 minimal medium and streaked onto M9 minimal agar plates supplemented with 19 amino acids (20 μg/ml) without tryptophan. Plates were incubated for 3–4 days at 30°C and growth was scored every 12 h.

2.4. Tryptophan synthetase assay

Overnight cultures of *E. coli* KS463 in M9 medium with or without tryptophan were inoculated on 400 ml of M9 as before. All cultures were incubated at 30°C to late log phase, harvested by centrifugation, washed twice with NaCl (0.9%), and resuspended in buffer A (0.05 M potassium phosphate (pH 7.0), 0.1 mg/ml of pyridoxal-5-phosphate, 10 mM 2-mercaptoethanol). Cell extract was prepared [14], dialyzed against buffer A containing 50% glycerol and stored at −20°C. IGP was freshly prepared as described [8]. Tryptophan synthetase was assayed in the IGP to Trp conversion with [^{3}H]serine (28.0 Ci/mmol) [15]. Enzymatic unit is defined as the amount producing 1 μmol of tryptophan/mg of protein.

3. Results

3.1. Formation of Glu-tRNA^{Glu} by heterologous GluRS

For this analysis we chose three heterologous *gltX* genes from *A. ferrooxidans* and *H. pylori*. *A. ferrooxidans* GluRS1 is known to glutamylate tRNA^{Glu} and the tRNA$^{Gln}_{CUG}$ isoacceptor, while GluRS2 preferentially acylates tRNA$^{Gln}_{UUG}$ [6]. The *H. pylori* GluRS2 is the special GluRS which recognizes only tRNA^{Gln} [5,6]. Recombinant plasmids carrying *A. ferrooxidans gltX1*, *gltX2* or *H. pylori gltX2* were transformed into *E. coli* strain JP1449. Cells transformed with recombinant plasmids proliferated normally at the permissive temperature (30°C) indicating that, in the presence of functional endogenous GluRS, the cloned genes were not toxic to *E. coli* (Fig. 1). At the non-permissive temperature (42°C) while *A. ferrooxidans gltX1* rescued the mutation in a similar way as did the homologous *E. coli gltX*, neither *A. ferrooxidans gltX2* nor *H. pylori gltX2* supported growth (Fig. 1). These results show that *gltX1*, but not *gltX2*, encodes a GluRS that generates *E. coli* Glu-tRNA^{Glu} in vivo.

3.2. Glu-tRNA^{Gln} is formed in vivo in E. coli

To demonstrate the in vivo formation of *E. coli* Glu-tRNA^{Gln}, we functionally replaced the inactive *trpA33* gene in the tryptophan auxotrophic strain *E. coli* KS463 by co-transformation of the cells with recombinant plasmids carrying *trpA49* either with glutamate (GAG) or glutamine (CAA or CAG) codons at position 49 of the TrpA protein. The formation of Glu-tRNA^{Gln} by the heterologous GluRS enzymes should lead to glutamate incorporation at the glutamine codon at position 49 of *trpA* restoring tryptophan synthetase activity, and thus prototrophy of cells. As shown in Fig. 2, *A. ferrooxidans* and *H. pylori gltX2* supported growth of the cells in the absence of tryptophan when *trpA49$_{CAA}$* variant was used. Neither *A. ferrooxidans gltX1* nor *E. coli gltX* were able to suppress the mutation. None of the heterologous *gltX* restored prototrophy to tryptophan of *trpA49$_{CAG}$* variant (data not shown). All transformed cells grew normally in the presence of tryptophan (data not shown). This implies that *A. ferrooxidans* and *H. pylori gltX2* encode GluRS enzymes that charge *E. coli* tRNA$^{Gln}_{UUG}$ species.

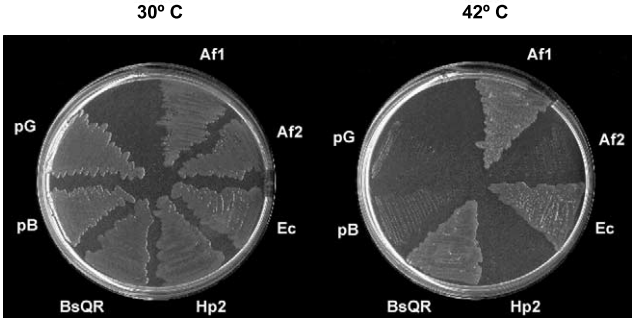

Fig. 1. Complementation of the *E. coli gltX^{ts}* strain JP1449 by heterologous *gltX* genes. Af1, *A. ferrooxidans gltX1*; Af2, *A. ferrooxidans gltX2*; Ec, *E. coli gltX*; Hp2, *H. pylori gltX2*; BsQR, *B. subtilis gltX* (Q373R); pB, pCBS1; pG, pGEX2t grown at 30 or at 42°C.

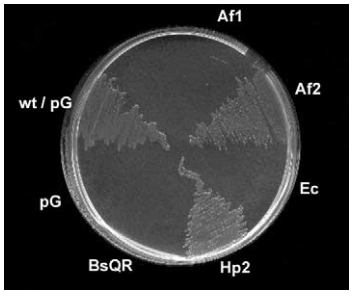

Fig. 2. Missense suppression of *E. coli trpA49$_{CAA}$* by heterologous *gltX* genes. *E. coli* strain KS463 was co-transformed with pMLC*trpA49$_{CAA}$* and *A. ferrooxidans gltX1* (Af1), *A. ferrooxidans gltX2* (Af2), *E. coli gltX* (Ec), *H. pylori gltX2* (Hp2), *B. subtilis gltX* Q373R (BsQR) or pGEX2t (pG). WT/pG, cells co-transformed with pMLC*trpA49*$^+$ and pGEX2t.

To confirm that restoration of prototrophy was due to the recovery of functional tryptophan synthetase, its enzymatic activity was measured in cells co-transformed with *trpA49$_{CAA}$* and *A. ferrooxidans gltX2* and compared to that from cells transformed with *trpA*$^+$ (Table 1). An eight-fold increase in the enzymatic activity was observed in sample cells compared to negative control (Table 1). The missense suppressed tryptophan synthetase activity was about 10% the activity of the wild-type control.

3.3. A single mutation makes B. subtilis gltX in vivo expression acceptable to E. coli

B. subtilis GluRS is a standard non-discriminating enzyme [3] and shown to glutamylate in vitro *E. coli* tRNA^{Glu} and the *E. coli* tRNA$^{Gln}_{UUG}$ [3]. The inability to overexpress *B. subtilis* GluRS in *E. coli* has been explained by the mischarging of this *E. coli* tRNA^{Gln} species [16].

Table 1
Recovery of tryptophan synthetase activity in cell extracts of *E. coli* KS463

	Af2/trpA49$_{CAA}$	*none/trpA*$^+$
Activity[a]	1.50 ± 0.25	15.7 ± 2.54

Tryptophan synthetase activity of extracts from KS463 cells co-transformed with *trpA49$_{CAA}$* and *A. ferrooxidans* gltX2 (*Af2*) and *trpA*$^+$ with empty vector (*none*) grown at 30°C. The tryptophan synthetase activity (0.187 ± 0.05) from cells co-transformed with *E. coli gltX* and *trpA49$_{CAA}$* grown with 0.01% tryptophan was subtracted from the values above.
[a]Units/mg protein.

More recently it was shown that a single amino acid change, R358Q, in *Thermus thermophilus* D-GluRS allowed in vitro charging of *E. coli* $tRNA^{Glu}$ containing a glutamine (CUG) anticodon [17]. Therefore we replaced the glutamine present in the corresponding position in *B. subtilis* GluRS with arginine to generate GluRS Q373R. The mutant *B. subtilis gltX* gene successfully rescued the thermosensitive GluRS mutation and allowed growth of *E. coli gltX* strain JP1449 at the non-permissive temperature (Fig. 1). Thus, *E. coli* Glu-$tRNA^{Glu}$ is generated by the mutant *B. subtilis* GluRS in vivo. However, the mutant *B. subtilis gltX* gene did not cause missense suppression of neither the *E. coli* $trpA49_{CAA}$ nor $trpA_{CAG}$ mutations, indicating that the mutant *B. subtilis* GluRS Q373R does not form Glu-$tRNA^{Gln}$.

Together, these results indicate that the Q373R replacement in *B. subtilis* GluRS restricted its tRNA specificity and eliminated charging of *E. coli* $tRNA^{Gln}_{UUG}$ in vivo.

4. Discussion

Missense suppression, the insertion of an incorrect amino acid for a certain codon, has been known for a long time [21], and work with the *trpA* system stimulated much molecular biology research (see e.g. [9]). As in a previous report for Asp/Asn [8], we have developed a system for the incorporation of glutamate in response to a glutamine (CAA) codon using mischarged Glu-$tRNA^{Gln}$ to generate wild-type tryptophan synthetase α-chain in a strain that is dependent on this enzyme for tryptophan synthesis. The required synthesis of an essential protein is the selective force that in this case leads to misincorporation of a canonical amino acid, and presumably forces the elongation factor EF-Tu to carry mischarged tRNA to the ribosome. Since this assumption is not in line with the current view of EF-Tu specificity [18–20], one may use this *trpA* system to probe the following questions: Will the ratio of EF-Tu and mischarged tRNA affect misincorporation? Compared to different amino acid pairs, do proteins tolerate more readily Glu/Gln exchanges as their physical size is similar (despite great functional difference)? Will the presence in *E. coli* of Glu-AdT 'detoxify' the *trpA* mischarging system? What are the molecular determinants that make a GluRS recognize $tRNA^{Gln}$? The *trpA* system will allow future studies to address these exciting questions.

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