Articles

Conserved Discrimination against Misacylated tRNAs by Two Mesophilic Elongation Factor Tu Orthologs[†]

Terry J. T. Cathopoulis,[‡] Pitak Chuawong,[‡,§] and Tamara L. Hendrickson*[,‡]

Departments of Chemistry, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, and Kasetsart University, Pahonyothin Road, Chatuchak, Bangkok 10900, Thailand

Received March 3, 2008; Revised Manuscript Received April 22, 2008

ABSTRACT: Elongation factor Tu (EF-Tu) binds and loads elongating aminoacyl-tRNAs (aa-tRNAs) onto the ribosome for protein biosynthesis. Many bacteria biosynthesize Gln-tRNA^{Gln} and Asn-tRNA^{Asn} by an indirect, two-step pathway that relies on the misacylated tRNAs Glu-tRNA^{Gln} and Asp-tRNA^{Asn} as intermediates. Previous thermodynamic and experimental analyses have demonstrated that *Thermus thermophilus* EF-Tu does not bind Asp-tRNA^{Asn} and predicted a similar discriminatory response against Glu-tRNA^{Gln} [Asahara, H., and Uhlenbeck, O. (2005) *Biochemistry 46*, 6194−6200; Roy, H., et al. (2007) *Nucleic Acids Res. 35,* 3420−3430]. By discriminating against these misacylated tRNAS, EF-Tu plays a direct role in preventing misincorporation of aspartate and glutamate into proteins at asparagine and glutamine codons. Here we report the characterization of two different mesophilic EF-Tu orthologs, one from *Escherichia coli*, a bacterium that does not utilize either Glu-tRNA^{Gln} or Asp-tRNA^{Asn}, and the second from *Helicobacter pylori*, an organism in which both misacylated tRNAs are essential. Both EF-Tu orthologs discriminate against these misacylated tRNAs, confirming the prediction that Glu-tRNA^{Gln}, like Asp-tRNA^{Asn}, will not form a complex with EF-Tu. These results also demonstrate that the capacity of EF-Tu to discriminate against both of these aminoacyl-tRNAs is conserved even in bacteria like *E. coli* that do not generate either misacylated tRNA.

By necessity, translation of the genetic code proceeds with high fidelity. This accuracy is ensured by a variety of mechanisms including high specificity in tRNA aminoacylation (*1*), editing of misacylated tRNAs (*2–4*), and proofreading of codon-anticodon interactions in the A-site of the ribosome (*3*). However, many bacteria are missing genes for glutaminyl- and/or asparaginyl-tRNA synthetase (GlnRS[1] and AsnRS, respectively), the enzymes that directly generate Gln-tRNA^{Gln} and Asn-tRNA^{Asn}. In these organisms, Gln-tRNA^{Gln} and Asn-tRNA^{Asn} are still obligate substrates for protein translation, but they are biosynthesized indirectly via parallel two-step processes that proceed through designed misacylation of these two tRNAs. First, a nondiscriminating glutamyl-tRNA synthetase (ND-GluRS) (*5, 6*), a tRNA^{Gln}-specific glutamyl-tRNA synthetase (GluRS2) (*7, 8*), or a nondiscriminating aspartyl-tRNA synthetase (ND-AspRS) (*9–12*) misacylates tRNA^{Gln} or tRNA^{Asn} with glutamate or aspartate: the resultant products are the misacylated tRNAs Glu-tRNA^{Gln} and Asp-tRNA^{Asn}, respectively. These misacylated tRNAs are subsequently converted to Gln-tRNA^{Gln} and Asn-tRNA^{Asn} by a glutamine-dependent Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase (Asp/Glu-Adt) (*13, 14*).

The use of misacylated Glu-tRNA^{Gln} and Asp-tRNA^{Asn} as essential aminoacyl-tRNA precursors necessitates the existence of proofreading mechanisms to prevent their fatal misuse in ribosomal protein synthesis. Asp/Glu-Adt and elongation factor Tu (EF-Tu), the G-protein responsible for loading aminoacyl-tRNAs onto the ribosome, have recently emerged as key players in the maintenance of translational accuracy (*15, 16*). In *Thermus thermophilus,* Asp/Glu-Adt forms a "transamidosome" complex with ND-AspRS and tRNA^{Asn}, but not with tRNA^{Asp}, to sequester Asp-tRNA^{Asn} and directly deliver it to Asp/Glu-Adt for repair (*17, 18*). The *T. thermophilus* ND-AspRS is an archaeal-type AspRS; the formation of a bacterial-type transamidosome, at least in a transient fashion, has been postulated and is supported

[†] This work was supported by a grant from the National Institutes of Health (GM071480). P.C. was supported by a DPST Fellowship from the Royal Thai Government.

* Corresponding author. Present address: Department of Chemistry, Wayne State University, 5101 Cass Ave, Detroit, MI 48202. Tel: 313-577-6914. Fax: 313-577-8822. E-mail: Tamara.hendrickson@chem.wayne.edu.

[‡] Johns Hopkins University.

[§] Kasetsart University.

[1] Abbreviations: AARS, aminoacyl-tRNA synthetase; AsnRS, asparaginyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; Asp/Glu-Adt, glutamine-dependent Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase; BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; EF-Tu, elongation factor Tu; GlnRS, glutaminyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; *Hp*, *Helicobacter pylori*; *H. pylori*, *Helicobacter pylori*; PEP, phosphoenolpyruvate; PK, pyruvate kinase; TCA, trichloroacetic acid. The 20 natural amino acids are referred to using the standard three-letter code.

10.1021/bi800369q CCC: $40.75
Published on Web 07/15/2008

by the observation that the affinity of Asp-tRNA^{Asn} for Asp/Glu-Adt is improved upon the addition of ND-AspRS to a transamidation reaction (*19*).

EF-Tu is responsible for loading nearly all elongating aminoacyl-tRNAs into the A site of the ribosome at the expense of GTP (*20*). (The one major exception is selenocysteinyl-tRNA^{Sec}, which has its own elongation factor (*21*)). Despite this broad specificity, EF-Tu does distinguish between different amino acids and tRNAs in its aminoacyl-tRNA substrates (*9, 15, 22–27*). Asp-tRNA^{Asn} shows little to no affinity to *Thermus thermophilus* EF-Tu·GTP under conditions where Asp-tRNA^{Asp} and Asn-tRNA^{Asn} each bind with low nanomolar K_d values (*28*). Binding of Glu-tRNA^{Gln} to *T. thermophilus* EF-Tu has not been directly measured but has been predicted to be ~2 kcal/mol less than that of Glu-tRNA^{Glu} (*24*). Formation of a *Pisum sativum* Glu-tRNA^{Gln}·EF-Tu·GTP complex does not occur with this organism's chloroplast EF-Tu; however, binding of this aa-tRNA was observed with the *E. coli* EF-Tu ortholog (*29*). Taken together, these experiments demonstrate that both Asp/Glu-Adt and EF-Tu participate in preventing Asp-tRNA^{Asn} and Glu-tRNA^{Gln} from entering the ribosome.

The role of EF-Tu as a proofreading protein has almost exclusively been studied using the thermophilic EF-Tu ortholog from *T. thermophilus*, an organism that utilizes GlnRS to directly generate Gln-tRNA^{Gln} but relies on indirect aminoacylation to generate Asp-tRNA^{Asn} en route to Asn-tRNA^{Asn} (*9*). Here we present the characterization of two mesophilic EF-Tu orthologs: the EF-Tu from *Escherichia coli*, an organism that uses GlnRS and AsnRS to directly generate both Gln-tRNA^{Gln} and Asn-tRNA^{Asn} (*30*) and consequently does not require proofreading of Glu-tRNA^{Gln} and Asp-tRNA^{Asn}, and the EF-Tu from *Helicobacter pylori*, a bacterium that lacks both GlnRS and AsnRS and consequently uses the Asp/Glu-Adt transamidation pathway for the biosynthesis of both Gln-tRNA^{Gln} and Asn-tRNA^{Asn} (*14, 31*). Each of these EF-Tu orthologs discriminated against both misacylated tRNAs, demonstrating that EF-Tu's role as a proofreading enzyme is conserved in mesophilic bacteria, independent of the method by which Asn-tRNA^{Asn} and Gln-tRNA^{Gln} are generated *in vivo*.

EXPERIMENTAL PROCEDURES

In Vivo Overexpression of ND-AspRS and Tryptophan Auxotrophy. The *E. coli trpA34* strain (provided by Professor Dieter Söll, Yale University) was transformed with either pQE-80L (empty vector) or pPTC001 (the plasmid encoding *H. pylori* ND-AspRS (*12*)). A single colony from each strain was used to inoculate a 200 mL overnight culture in M9 minimal media supplemented with 20 amino acids (20 μg/mL) and 0.5% glucose as a carbon source. The overnight culture was harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. The cell pellets were gently washed twice with 200 mL M9 minimal media supplemented with 0.5% glucose and each encoded amino acid (20 μg/mL) except tryptophan. The cell pellets were then gently resuspended in the same media. These cell suspensions were used to streak M9 minimal media agar plates supplemented with 0.5% glucose, 19 amino acids (20 μg/mL), in the absence and presence of tryptophan, and 0, 0.1, or 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Plates were incubated at 37 °C and the growth of cells was assessed after 24 h.

Overexpression and Purification of H. pylori Aminoacyl-tRNA Synthetases. H. pylori GluRS1, GluRS2, and ND-AspRS were overexpressed and purified as previously described (*8, 12*).

Cloning, Overexpression, and Purification of E. coli and H. pylori EF-Tu·GDP. The plasmid encoding a six histidine tagged *E. coli* EF-Tu construct (*32*) was obtained from Professor David Draper (Johns Hopkins University). The gene encoding *H. pylori* EF-Tu (*tufB*, Hp1205) was amplified from *H. pylori* strain 26695 chromosomal DNA (ATCC) using the following primers: TLH-EFTU-A (5′-ggagaa**ggatcc**atggcaaaagaaaagtttaacagaactaagcc) and TLH-EFTU-B (5′-ttttt**gaagctt**ttattcaataatattgctcacaacacc); these primers introduced *Bam*HI and *Hin*dIII sites (bold) onto the 5′ and 3′ ends of the *tufB* gene. The resultant product was cloned into the *Bam*HI and *Hin*dIII sites of pQE-80L (Qiagen). This vector (pPTC033) introduces an N-terminal 6-histidine tag onto the encoded protein.

Both EF-Tu orthologs were purified using Ni-NTA spin columns (Qiagen), adjusting the manufacturer's instructions so that all buffers contained 50 μM guanosine diphosphate (GDP). Eluted EF-Tu was concentrated using microcon spin filters (Millipore) and exchanged into buffer containing 50 mM Tris·HCl (pH 7.5), 25 mM KCl, 15 mM $MgCl_2$, 2 mM β-mercaptoethanol, 50 μM GDP (*33*). EF-Tu was stored at −20 °C in the same buffer with 50% glycerol.

In Vivo Expression of H. pylori tRNAs and tRNA Purification. Each *H. pylori* tRNA was overexpressed *in vivo* in *E. coli* as previously described (*8, 12*). Small RNAs were purified using the Nucleobond RNA/DNA Maxi Kit (Clontech), according to the manufacturer's instructions. This procedure produces a mixture of tRNAs that is enriched with the *H. pylori* tRNA of interest but also contains total *E. coli* tRNA as well as other small RNAs. Yields of a given tRNA isoacceptor were quantified by aminoacylation by GluRS1, GluRS2, or ND-AspRS (see below). Typical yields ranged from 500 to 1600 pmol per OD_{260}. *Ec* AspRS was used to verify the amount of *Ec* tRNA^{Asp} in samples of overexpressed *Hp* tRNA^{Asn}. (*Ec* AspRS-expressing strain was a gift of Rachel Green, Johns Hopkins University.)

Transfer RNA Aminoacylation. Prior to aminoacylation, the five different *H. pylori* tRNAs (tRNA^{Glu1}, tRNA^{Glu2}, tRNA^{Gln}, tRNA^{Asp} and tRNA^{Asn}) were adjusted by the addition of batch *E. coli* tRNA (Sigma-Aldrich) to similar levels of purity (~500 pmol/OD). *H. pylori* GluRS1 was used to aminoacylate tRNA^{Glu1} and tRNA^{Glu2}; *H. pylori* GluRS2 was used to aminoacylate tRNA^{Gln}; and *H. pylori* ND-AspRS was used to aminoacylate tRNA^{Asp} and tRNA^{Asn}. Each tRNA (~20−100 μM) was incubated for 1 h at 37 °C in 10 mM Hepes·OH (pH 7.5), with 2 mM ATP, and 8 mM $MgCl_2$, glutamic acid or aspartic acid, and 1 μM enzyme. The amino acid concentrations were varied as needed for downstream experiments and ranged from 2 to 200 μM. For assays with low levels of amino acid, commercial ^{3}H-[3,4]-glutamic acid (Perkin-Elmer, 45.4 Ci/mmol) or ^{3}H-[2,3]-aspartic acid (Amersham, 34 Ci/mmol) was used without subsequent dilution. For higher amino acid concentration assays, unlabeled amino acid was used alone or supplemented with trace levels of radioactivity.

Column Chromatography Assay. Binding of different aminoacyl-tRNAs to EF-Tu was initially analyzed using a Ni^{2+} affinity column, essentially as previously described (*9*).

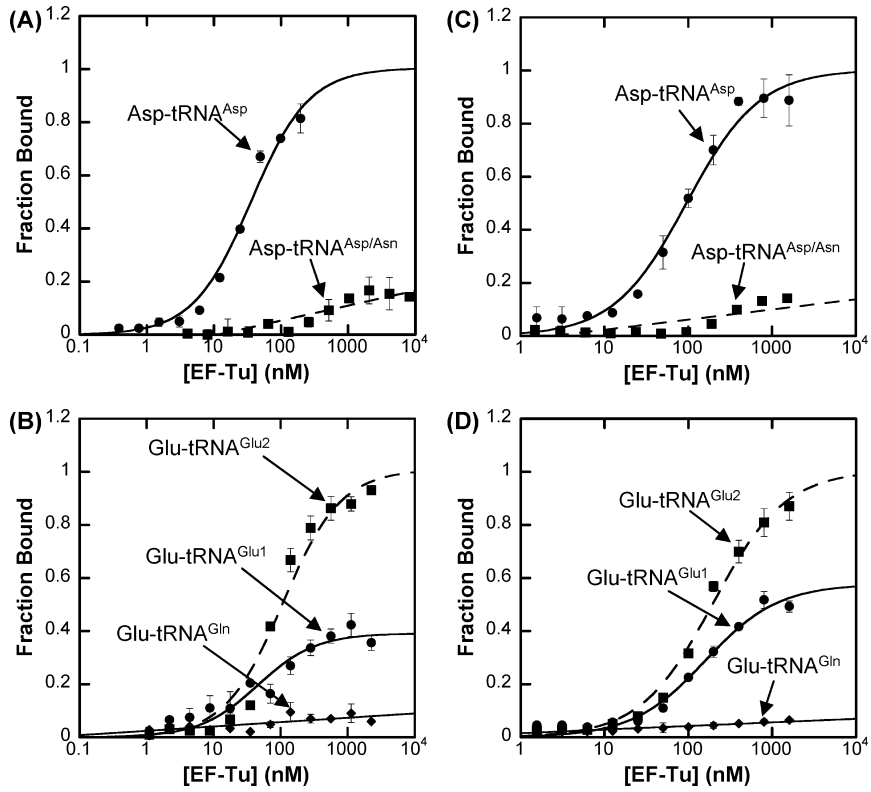

FIGURE 1: EF-Tu discriminates against *H. pylori* Asp-tRNA^{Asn} and Glu-tRNA^{Gln}. Panels A−D show the average of triplicate RNase protection assays; the error bars represent standard error. (A) *E. coli* EF-Tu with *H. pylori* Asp-tRNA^{Asp} (circles) and Asp-tRNA^{Asn} (squares); (B) *E. coli* EF-Tu with *H. pylori* Glu-tRNA^{Glu1} (circles), Glu-tRNA^{Glu2} (squares), and Glu-tRNA^{Gln} (diamonds); (C) *H. pylori* EF-Tu with *H. pylori* Asp-tRNA^{Asp} (circles) and Asp-tRNA^{Asn} (squares); (D) *H. pylori* EF-Tu with *H. pylori* Glu-tRNA^{Glu1} (circles), Glu-tRNA^{Glu2} (squares), and Glu-tRNA^{Gln} (diamonds).

Prior to column preparation, tRNA aminoacylation reactions were conducted as described above using 2−3 μM ^{3}H-amino acid (undiluted). Columns were loaded with 400 μL of Ni-NTA slurry (Qiagen) and washed with 1 mL of buffer A (50 mM Tris·Cl, 10 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM KCl, 5 mM β-mercaptoethanol, 15 mM guanosine triphosphate (GTP), 15 mM phosphoenolpyruvate (PEP)). EF-Tu·GDP (1400 pmol in 200 μL) was added to the resin, and buffer A (200 μL) was added; columns were agitated at 4 °C for one hour. After EF-Tu binding, buffer was allowed to flow through. EF-Tu·GDP was then converted to EF-Tu·GTP by incubation for 30 min at room temperature in 200 μL of buffer B (buffer A supplemented with 0.3 mg/mL pyruvate kinase (PK)). After activation, buffer was allowed to flow through. Columns were then treated with 1−7 pmol of ^{3}H-aa-tRNA in buffer B (200 μL final volume) and agitated at room temperature for 30 min. The total concentration of EF-Tu on these columns is 3.5 μM based on total binding of 1400 pmol of EF-Tu and the combined volume of 200 μL of resin and 200 μL of aa-tRNA-containing buffer; because EF-Tu is resin-bound, local concentrations are likely to be higher. Initial flow through was collected for quantification of unbound aa-tRNA. Columns were washed ten times each with 200 μL of buffer A. Columns were subsequently treated five times each with 200 μL of buffer A, supplemented with 0.1 M NaCl. Bound ^{3}H-aa-tRNA was eluted by treating columns seven times with 200 μL of buffer C (0.1 M sodium borate (pH 7.5), 1 M NaCl). All fractions were quantified by liquid scintillation. Reported data (Figure 2) represents the average of experiments run in triplicate and the error bars reflect standard error.

Activation of EF-Tu. EF-Tu·GDP was converted to EF-Tu·GTP according to protocols previously reported (*34*). Briefly, EF-Tu (5 − 15 μM as determined by Bradford assay (Biorad)) was agitated at room temperature in 50 mM Hepes (pH 7.0), NH_4Cl (150 mM for RNase protection assay and 50 mM for deacylation assays), 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20 μM GTP, 3 mM PEP, 50 μg/mL PK. EF-Tu·GTP was kept on ice and used immediately.

The concentration of active EF-Tu·GTP (defined as protein capable of binding aa-tRNAs) was determined using an RNase protection assay modified from that previously described (*35*). EF-Tu·GTP (∼5 μM as determined by Bradford assay) was incubated at 4 °C for 30 min (50 μL total volume) under buffer conditions described above supplemented with either *H. pylori* ^{3}H-Asp-tRNA^{Asp} (34 Ci/mmol) or ^{3}H-Glu-tRNA^{Glu1} (45.4 Ci/mmol); the final concentration of each aa-tRNA was 15−20 μM. RNase A (Sigma-Aldrich) was added to a final concentration of 0.01 mg/mL, and the reaction was incubated at 4 °C for 20 s. The reaction was quenched with 100 μL 10% trichloroacetic acid (TCA, Fisher) that had been supplemented with 0.1 mg/mL total *E. coli* tRNA (Sigma-Aldrich). These assays were conducted in triplicate. An aliquot of each reaction mixture (100 μL) was loaded onto a 2.5 cm filter (Whatman) that had been saturated with 200 μL of 5% TCA. Filters were washed 3 × 15 min in 5% TCA followed by a single 5 min wash in EtOH. Pads were dried and soaked in 3 mL of Ecolite + Scintillation cocktail (Mp Biomedicals), and the amount of intact aa-tRNA was quantified using an LS6500 Scintillation counter (Beckman). These data were corrected

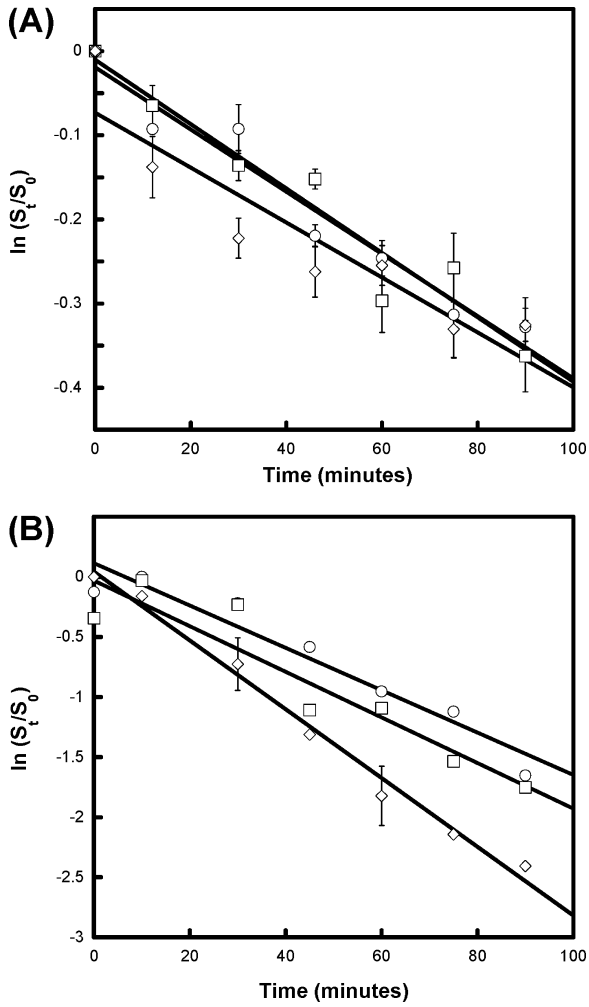

FIGURE 2: EF-Tu does not protect Asp-tRNA^{Asn} or Glu-tRNA^{Gln} from deacylation. (A) Impact of EF-Tu on deacylation rates for Asp-tRNA^{Asn}. Asp-tRNA^{Asn} + *E. coli* EF-Tu (circles); Asp-tRNA^{Asn} + *H. pylori* EF-Tu (squares); Asp-tRNA^{Asn} alone (diamonds). (B) Impact of EF-Tu on deacylation rates for Glu-tRNA^{Gln}. Glu-tRNA^{Gln} + *E. coli* EF-Tu (circles); Glu-tRNA^{Gln} + *H. pylori* EF-Tu (squares); Glu-tRNA^{Gln} alone (diamonds). Data is plotted according to the equation $\mathrm{Ln}(S_t/S_0 = -kt)$, where S_t equals the concentration of aa-tRNA at a given time, t; S_0 equals the initial concentration of aa-tRNA; and k equals the rate of hydrolysis. Data shown is from the average of experiments run in triplicate; error bars represent standard errors. See Supporting Information for deacylation assays with Asp-tRNA^{Asp}, Glu-tRNA^{Glu1}, and Glu-tRNA^{Glu2}.

for the amount of intact aminoacylated-tRNA remaining in a control reaction where the EF-Tu was omitted.

The active concentration of *H. pylori* EF-Tu was typically ~10% of that predicted by Bradford assay, whereas that of *E. coli* EF-Tu was typically ~40% of that observed by Bradford assay. The percent active EF-Tu was independent of the nature of the cognate aminoacyl-tRNA (Asp-tRNA^{Asp} versus Glu-tRNA^{Glu1}). This enigmatic disparity between the level of active EF-Tu and amount of protein present is a well-documented phenomenon (*27, 36, 37*). Unless otherwise stated, all reported EF-Tu·GTP concentrations are based on the results of this binding assay.

RNase Protection Assay. Dissociation constants were determined for each *H. pylori* aa-tRNA using a protocol adapted from that developed by Uhlenbeck and colleagues (*27, 34*). A stock of active EF-Tu·GTP was prepared using the above procedure in the presence of 150 mM NH_4Cl. Twelve concentrations of EF-Tu·GTP (25 μL each, ranging in concentration from ~1 nM to 2 mM) were prepared by serial dilution in a 96-well plate at 4 °C. To each well, aa-tRNA was added (25 μL, ~ 20 nM final concentration in 150 mM Hepes (pH 7.0), 150 mM NH_4Cl, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20 μM GTP, 3 mM PEP, 50 μg/mL PK). After incubation for 30 min, RNase A was added to a final concentration of 0.01 μg/mL. After a 20 s incubation on ice, aa-tRNA degradation was quenched by the addition of 100 μL of 10% TCA that had been supplemented with 0.1 mg/mL unfractionated *E. coli* tRNA (Sigma-Aldrich). The amount of intact aa-tRNA was quantified as described above. Data was analyzed using Kaleidagraph v. 3.6.2. K_d values were determined by plotting the concentration of EF-Tu·GTP·aa-tRNA complex against the initial concentration of EF-Tu·GTP and applying the formula $[\mathrm{complex}] = ((K_d + [\mathrm{EF\text{-}Tu}]_i + [\mathrm{aa\text{-}tRNA}]_i) - ((K_d + [\mathrm{EF\text{-}Tu}]_i + [\mathrm{aa\text{-}tRNA}]_i)^2 - (4 \times [\mathrm{aa\text{-}tRNA}]_i \times [\mathrm{EF\text{-}Tu}]_i))^{0.5})/2$. All experiments were conducted in triplicate, and error bars represent standard error from these replicates.

Deacylation Protection Assay. A stock of active EF-Tu·GTP was prepared using the above procedure in the presence of 50 mM NH_4Cl. Reactions containing 1 μM active EF-Tu·GTP and 50 nM ^{3}H-aa-tRNA in 50 mM Hepes (pH 7.0), 50 mM NH_4Cl, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20 μM GTP, 3 mM PEP, 50 μg/mL PK were incubated at 4 °C for 30 min. Solutions were transferred to 37 °C, and the rate of deacylation was determined by quenching aliquots (10 μL) at various times over 90 min. Each time point was quenched onto pads prepared with 5% TCA and counted as described above to assess the degree of deacylation.

Table 1: Physical constants for *E. coli* and *H. pylori* EF-Tu with different *H. pylori* aa-tRNAs

	E. coli EF-Tu		*H. pylori* EF-Tu	
	K_d (nM)[a]	$\Delta G°$ (kcal/mol)[b]	K_d (nM)	$\Delta G°$ (kcal/mol)
Asp-tRNA^{Asp}	34 ± 6	−9.4	92 ± 13	−8.9
Asp-tRNA^{Asn}	>1600	>−7.3	>1600	>−7.3
Glu-tRNA^{Glu1}	46 ± 13	−9.3	155 ± 26	−8.6
Glu-tRNA^{Glu2}	96 ± 18	−8.9	181 ± 22	−8.5
Glu-tRNA^{Gln}	>1600	>−7.3	>1600	>−7.3

[a] Errors are presented as standard errors from experiments run in triplicate. [b] $\Delta G° = -RT \ln(1/K_d)$.

RESULTS

Neither H. pylori nor E. coli EF-Tu Binds H. pylori Asp-tRNA^{Asn} or Glu-tRNA^{Gln} as Strongly as They Bind Asp-tRNA^{Asp} and Glu-tRNA$^{Glu1/2}$. A ribonuclease (RNase) protection assay (*27, 34*) was used to determine apparent dissociation constants (K_d) between each of the two noncognate and three cognate aa-tRNAs and EF-Tu·GTP (Figure 1 and Table 1). As expected, each cognate aa-tRNA bound both EF-Tu orthologs with high affinity. The *E. coli* EF-Tu revealed tighter K_d values ranging from about 35 to 95 nM, whereas those for the *H. pylori* EF-Tu ranged from about 90 to 180 nM. (For reasons not well understood, only about 35% of the Glu-tRNA^{Glu1} was protected in this RNase assay. However, complete complex formation was observed using a deacylation assay, see Figure S1.) When the two misacylated tRNAs were examined neither bound significantly to

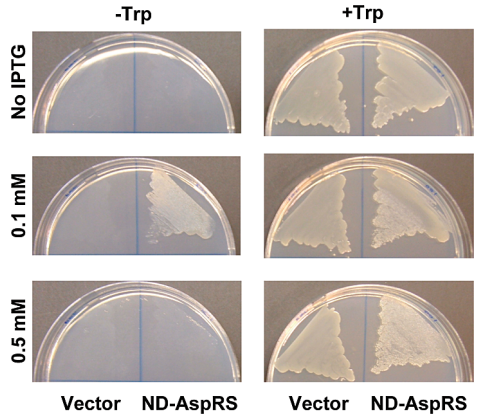

FIGURE 3: Expression of *H. pylori* ND-AspRS in *E. coli* suppresses tryptophan auxotrophy. *E. coli trpA34* cells containing a plasmid expressing either *H. pylori* ND-AspRS (pPTC001) or empty vector (pQE-80L) were streaked on plates in the absence (left) or presence (right) of tryptophan and with varying concentrations of IPTG. Low levels of ND-AspRS expression (0.1 mM IPTG) suppressed tryptophan auxotrophy.

either EF-Tu ortholog. (Analysis with *Ec* D-AspRS confirmed that the low levels of complex formation observed with Asp-tRNA^{Asn} (Figure 1) are due to contaminating *E. coli* Asp-tRNA^{Asp}, data not shown.) In fact, neither ortholog was capable of protecting even 5% of the Asp-tRNA^{Asn} or Glu-tRNA^{Gln} present in each experiment, even at the highest concentration examined (~1.6−3 μM EF-Tu). While these observations are consistent with previous *in vitro* experiments showing that *E. coli* EF-Tu can discriminate against *T. thermophilus* Asp-tRNA^{Asn}, this is the first example of a quantitative *in vitro* assay demonstrating a similar discrimination against Glu-tRNA^{Gln} (*9, 28*).

EF-Tu Does Not Prevent Hydrolysis of Either Asp-tRNA^{Asn} or Glu-tRNA^{Gln}. Deacylation rates were examined for each of the five *H. pylori* aa-tRNAs being evaluated herein in the absence of protein and in the presence of either *E. coli* or *H. pylori* EF-Tu·GTP (Figure 2 and Figure S1). Aminoacyl-tRNAs are inherently labile under aqueous conditions, and this spontaneous hydrolysis is suppressed when an aa-tRNA is bound to EF-Tu (*38*). For this reason, deacylation assays offer a method for examining complex formation between EF-Tu and various aa-tRNAs that is orthogonal to the RNase protection assay described above. This method offers the additional advantage that it does not rely on the action of an additional enzyme (e.g., RNase). The results of these experiments recapitulate those from the RNase protection assay: The three accurately aminoacylated aa-tRNAs were protected from deacylation in the presence of either EF-Tu ortholog (Figure S1); however, the addition of EF-Tu did not protect either Asp-tRNA^{Asn} or Glu-tRNA^{Gln} from hydrolysis (Figure 2).

Overexpression of H. pylori ND-AspRS in E. coli Results in Aspartate Incorporation at Asparagine Codons. It has previously been shown that overexpression of *B. subtilis* ND-AspRS leads to suppression of tryptophan auxotrophy in the *E. coli* strain *trpA34* (*39*). This strain carries a GAT → AAT mutation at codon 60 of the *trpA* gene, introducing an Asp60Asn mutation into the α-subunit of tryptophan synthase and inactivating the enzyme; consequently, this strain requires exogenous tryptophan for viability (*40*). Because ND-AspRS can aminoacylate *E. coli* tRNA^{Asn} with aspartate, expression of *B. subtilis* ND-AspRS facilitated the incorporation of aspartate into tryptophan synthase at position 60, via ribosomally accessible Asp-tRNA^{Asn}, at sufficient levels to restore tryptophan synthase activity and viability in the absence of tryptophan (*39*). This observation is in apparent contradiction with the results described above which show that *E. coli* EF-Tu·GTP does not bind Asp-tRNA^{Asn}.

We similarly tested the overexpression of *H. pylori* ND-AspRS in *trpA34* under varying conditions of induction. Tryptophan auxotrophy was suppressed specifically within a narrow range of IPTG concentrations (Figure 3). No growth was observed in the absence of tryptophan and IPTG (top left panel). When 0.1 mM IPTG was added, sufficient Asp-tRNA^{Asn} was used in the biosynthesis of tryptophan synthase to suppress the requirement for tryptophan (middle left panel). At higher IPTG concentrations, lethality in the absence of tryptophan was restored, suggesting that levels of aspartate incorporation into proteins at asparagine codons had become too high to promote viability (left bottom panel). In the presence of tryptophan, these strains were all viable under the conditions screened (right panels). Interestingly, the strains grown in the presence of tryptophan were viable in the presence of 0.5 mM IPTG: a level high enough to introduce ND-AspRS toxicity in the -Trp counterpart. This viability is thought to be due to a higher resilience afforded by the healthier environment of the tryptophan enriched plates.

High Local Concentrations of H. pylori or E. coli EF-Tu Induces Binding of Asp-tRNA^{Asn} and Glu-tRNA^{Gln}. The observation that expression of *H. pylori* ND-AspRS can suppress tryptophan auxotrophy in the *trpA34* strain demonstrates that this enzyme misacylates *E. coli* tRNA^{Asn} *in vivo* and that *E. coli* EF-Tu can bind the resultant Asp-tRNA^{Asn} at levels that are sufficient to promote errors in protein biosynthesis under selective conditions (similar to previous results with *B. subtilis* ND-AspRS (*39*)). Other complementation studies have also demonstrated *in vivo* incorporation of glutamate at glutamine codons via Glu-tRNA^{Gln} (*41*). To resolve these results with our quantitative demonstration that the *E. coli* EF-Tu·GTP discriminates against Asp-tRNA^{Asn} and Glu-tRNA^{Gln}, we sought to identify conditions where binding of Asp-tRNA^{Asn} and/or Glu-tRNA^{Gln} to EF-Tu·GTP could be recapitulated *in vitro*. The *E. coli* and *H. pylori* EF-Tu orthologs were both examined in order to determine whether differences exist in organisms that misacylate tRNA^{Asn} and tRNA^{Gln} (*H. pylori*) versus organisms that accurately aminoacylate these tRNAs directly (*E. coli*).

Each relevant aminoacylated *H. pylori* tRNA (Asp-tRNA^{Asp}, Asp-tRNA^{Asn}, Glu-tRNA^{Glu1}, Glu-tRNA^{Glu2}, and Glu-tRNA^{Gln}, each with a radiolabel incorporated into the amino acid) was separately loaded onto a Ni^{2+} column containing either 6-His tagged *E. coli* EF-Tu·GTP or *H. pylori* EF-Tu·GTP at high local concentrations (≥3.5 μM). Based on a previously published approach (*9*), the columns were washed and then bound aa-tRNAs were eluted by treatment with high salt. Consistent with the *in vivo* tryptophan auxotrophy experiments, all five different *H. pylori* aa-tRNAs, including the two misacylated tRNAs, bound both EF-Tu orthologs under the conditions tested (Figure 4). Asp-tRNA^{Asp} and Asp-tRNA^{Asn} bound each EF-Tu at essentially identical levels (~80% bound); in contrast,

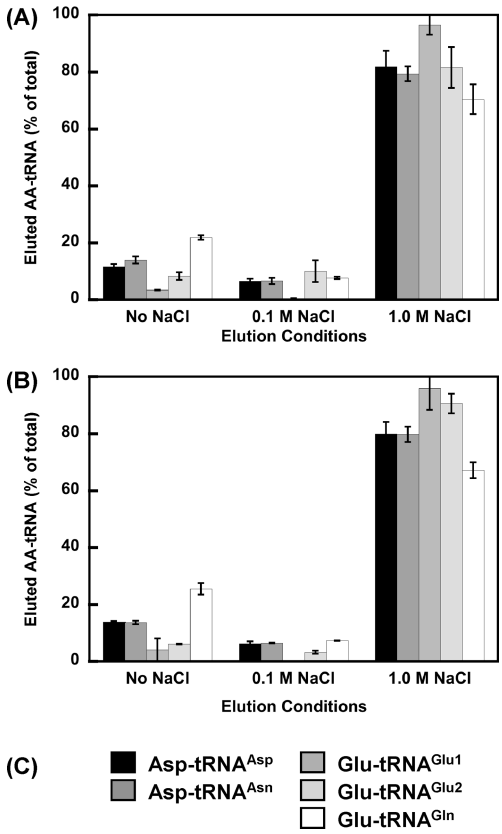

FIGURE 4: EF-Tu binds Asp-tRNA^{Asn} and Glu-tRNA^{Gln}. (A) *H. pylori* and (B) *E. coli* EF-Tu were each tested for binding to five different tRNAs (Asp-tRNA^{Asp}, Asp-tRNA^{Asn}, Glu-tRNA^{Glu1}, Glu-tRNA^{Glu2}, and Glu-tRNA^{Gln}) using a nickel affinity column and high salt elution of bound aa-tRNAs (*9*). (C) Shading scheme used in panels A and B. Data shown is from the average of experiments run in triplicate; error bars represent standard error.

Glu-tRNA^{Gln} did not bind either EF-Tu to the same extent as the two Glu-tRNA^{Glu} acceptors (~70% bound versus 80−95% bound, respectively).

DISCUSSION

We have demonstrated herein that two different mesophilic EF-Tu orthologs, one from *H. pylori* and one from *E. coli*, bind the accurately aminoacylated tRNAs Asp-tRNA^{Asp}, Glu-tRNA^{Glu1}, and Glu-tRNA^{Glu2} with low nanomolar affinities as expected. Under these same conditions, binding was not observed for either Asp-tRNA^{Asn} or Glu-tRNA^{Gln}, the two misacylated tRNAs that are essential for *H. pylori* viability but not for *E. coli*. These two noncognate species do exhibit a capacity to complex with EF-Tu under non-native conditions (e.g., overexpression of AspRS, Figure 3, or high local concentrations of EF-Tu, Figure 4). This last result (Figure 4) explains the discrepancy between *in vivo* experiments reported herein (Figure 3) and by Söll and colleagues (*39, 40*), which demonstrate formation of functional EF-Tu·GTP·Asp-tRNA^{Asn}/Glu-tRNA^{Gln} complexes *in vivo*, and *in vitro* experiments (Figures 1 and 2 and references *9, 28, 29*), which show that EF-Tu·GTP discriminates against Asp-tRNA^{Asn} and Glu-tRNA^{Gln} in favor of accurately aminoacylated tRNAs. Clearly, endogenous levels of the aminoacyl-tRNA synthetases and EF-Tu are carefully balanced *in vivo* to prevent catastrophic errors. Perturbation of this system via overexpression of a nondiscriminating aminoacyl-tRNA synthetase, in combination with the high *in vivo* concentration of EF-Tu (~170 μM (*42*)), ablates the proofreading capacity of EF-Tu such that Asp-tRNA^{Asn} and Glu-tRNA^{Gln} are incorrectly taken into the ribosome at levels sufficient for complementation.

The observation that the two EF-Tu orthologs examined herein discriminate against Asp-tRNA^{Asn} is consistent with work prior to the present study that had demonstrated that the *T. thermophilus* and *E. coli* EF-Tu orthologs both differentiate against this misaspartylated species in favor of Asp-tRNA^{Asp} and Asn-tRNA^{Asn} (*28*). With respect to Glu-tRNA^{Gln}, researchers have previously calculated that EF-Tu would be incapable of binding this misacylated species (*24*) (a result qualitatively observed with the chloroplast EF-Tu from *P. sativum* (*29*)). Here we have quantitatively demonstrated that both the *E. coli* and *H. pylori* EF-Tu orthologs do indeed discriminate against this misacylated tRNA, with complex formation being disfavored by at least 2 kcal/mol compared to accurately aminoacylated tRNAs. In total, the results herein further support the hypothesis that EF-Tu has universally retained the ability to prevent the endogenously produced, potentially toxic, misacylated tRNAs Asp-tRNA^{Asn} and Glu-tRNA^{Gln} from entering ribosomal translation. The observation that an organism such as *E. coli*, that contains no biochemical means of producing either misacylated tRNA, exhibits this vestigial proofreading capacity suggests that this discrimination mechanism existed in the last common universal ancestor along with the indirect transamidation pathway for Asn-tRNA^{Asn} and Gln-tRNA^{Gln} biosynthesis.

ACKNOWLEDGMENT

The authors thank Professor Dieter Söll (Yale University) for providing a sample of the *trpA34* strain, Rachel Green (Johns Hopkins Medical School) for the Ec D-AspRS express in strain, Marc Greenberg and David Draper (Johns Hopkins University) for generous access to their equipment, and David Draper (JHU), Olke Uhlenbeck, and Lee Sanderson (Northwestern University) for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Data outlining the deacylation rates of Glu-tRNA^{Glu1}, Glu-tRNA^{Glu2} and Asp-tRNA^{Asp}. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Ibba, M., and Soll, D. (2000) Aminoacyl-tRNA synthesis. *Annu. Rev. Biochem. 69*, 617–650.
2. Hendrickson, T. L., and Schimmel, P. (2003) Transfer RNA-Dependent Editing by Aminoacyl-tRNA Synthetases, in *Translation Mechanisms* (Lapointe, J., Ed.) pp 34−64, Kluwer Academic Press, New York.
3. Cochella, L., and Green, R. (2005) Fidelity in protein synthesis. *Curr. Biol. 15*, R536–540.
4. Mascarenhas, A., Martinis, S., An, S., Rosen, A., and Musier-Forsyth, K. (2007) Fidelity mechanisms of the aminoacyl-tRNA synthetases, in *Protein Engineering* (RajBhandary, U. L., and Koehrer, C., Eds.) Springer-Verlag, New York.
5. Lapointe, J., Duplain, L., and Proulx, M. (1986) A single glutamyl-tRNA synthetase aminoacylates tRNA^{Glu} and tRNA^{Gln} in Bacillus subtilis and efficiently misacylates *Escherichia coli* tRNA^{Gln1} in vitro. *J. Bacteriol. 165*, 88–93.
6. Schulze, J. O., Masoumi, A., Nickel, D., Jahn, M., Jahn, D., Schubert, W. D., and Heinz, D. W. (2006) Crystal structure of a non-discriminating glutamyl-tRNA synthetase. *J. Mol. Biol. 361*, 888–897.

7. Salazar, J. C., Ahel, I., Orellana, O., Tumbula-Hansen, D., Krieger, R., Daniels, L., and Soll, D. (2003) Coevolution of an aminoacyl-tRNA synthetase with its tRNA substrates. *Proc. Natl. Acad. Sci. U.S.A. 100*, 13863–13868.
8. Skouloubris, S., Ribas de Pouplana, L., De Reuse, H., and Hendrickson, T. L. (2003) A noncognate aminoacyl-tRNA synthetase that may resolve a missing link in protein evolution. *Proc. Natl. Acad. Sci. U.S.A. 100*, 11297–11302.
9. Becker, H. D., and Kern, D. (1998) Thermus thermophilus: a link in evolution of the tRNA-dependent amino acid amidation pathways. *Proc. Natl. Acad. Sci. U.S.A. 95*, 12832–12837.
10. Charron, C., Roy, H., Blaise, M., Giege, R., and Kern, D. (2003) Non-discriminating and discriminating aspartyl-tRNA synthetases differ in the anticodon-binding domain. *EMBO J. 22*, 1632–1643.
11. Bernard, D., Akochy, P. M., Beaulieu, D., Lapointe, J., and Roy, P. H. (2006) Two residues in the anticodon recognition domain of the aspartyl-tRNA synthetase from *Pseudomonas aeruginosa* are individually implicated in the recognition of tRNAAsn. *J. Bacteriol. 188*, 269–274.
12. Chuawong, P., and Hendrickson, T. L. (2006) The nondiscriminating aspartyl-tRNA synthetase from *Helicobacter pylori*: anticodon-binding domain mutations that impact tRNA specificity and heterologous toxicity. *Biochemistry 45*, 8079–8087.
13. Curnow, A. W., Hong, K., Yuan, R., Kim, S., Martins, O., Winkler, W., Henkin, T. M., and Soll, D. (1997) Glu-tRNA^{Gln} amidotransferase: a novel heterotrimeric enzyme required for correct decoding of glutamine codons during translation. *Proc. Natl. Acad. Sci. U.S.A. 94*, 11819–11826.
14. Sheppard, K., Akochy, P. M., Salazar, J. C., and Soll, D. (2007) The Helicobacter pylori amidotransferase GatCAB is equally efficient in glutamine-dependent transamidation of Asp-tRNA^{Asn} and Glu-tRNA^{Gln}. *J. Biol. Chem. 282*, 11866–11873.
15. LaRiviere, F. J., Wolfson, A. D., and Uhlenbeck, O. C. (2001) Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. *Science 294*, 165–168.
16. Dale, T., and Uhlenbeck, O. C. (2005) Amino acid specificity in translation. *Trends Biochem. Sci. 30*, 659–665.
17. Bailly, M., Blaise, M., Roy, H., Deniziak, M., Lorber, B., Birck, C., Becker, H. D., and Kern, D. (2008) tRNA-dependent asparagine formation in prokaryotes: Characterization, isolation and structural and functional analysis of a ribonucleoprotein particle generating Asn-tRNA(Asn). *Methods 44*, 146–163.
18. Bailly, M., Blaise, M., Lorber, B., Becker, H. D., and Kern, D. (2007) The transamidosome: a dynamic ribonucleoprotein particle dedicated to prokaryotic tRNA-dependent asparagine biosynthesis. *Mol. Cell 28*, 228–239.
19. Huot, J. L., Balg, C., Jahn, D., Moser, J., Emond, A., Blais, S. P., Chenevert, R., and Lapointe, J. (2007) Mechanism of a GatCAB amidotransferase: aspartyl-tRNA synthetase increases its affinity for Asp-tRNA(Asn) and novel aminoacyl-tRNA analogues are competitive inhibitors. *Biochemistry 46*, 13190–13198.
20. Rodnina, M. V., Gromadski, K. B., Kothe, U., and Wieden, H. J. (2005) Recognition and selection of tRNA in translation. *FEBS Lett. 579*, 938–942.
21. Copeland, P. R. (2003) Regulation of gene expression by stop codon recoding: selenocysteine. *Gene 312*, 17–25.
22. Wagner, T., and Sprinzl, M. (1980) The complex formation between *Escherichia coli* aminoacyl-tRNA, elongation factor Tu and GTP. The effect of the side-chain of the amino acid linked to tRNA. *Eur. J. Biochem. 108*, 213–221.
23. Asahara, H., and Uhlenbeck, O. C. (2002) The tRNA specificity of *Thermus thermophilus* EF-Tu. *Proc. Natl. Acad. Sci. U.S.A. 99*, 3499–3504.
24. Asahara, H., and Uhlenbeck, O. C. (2005) Predicting the binding affinities of misacylated tRNAs for *Thermus thermophilus* EF-Tu.GTP. *Biochemistry 44*, 11254–11261.
25. Dale, T., Sanderson, L. E., and Uhlenbeck, O. C. (2004) The affinity of elongation factor Tu for an aminoacyl-tRNA is modulated by the esterified amino acid. *Biochemistry 43*, 6159–6166.
26. Sanderson, L. E., and Uhlenbeck, O. C. (2007) Exploring the specificity of bacterial elongation factor Tu for different tRNAs. *Biochemistry 46*, 6194–6200.
27. Sanderson, L. E., and Uhlenbeck, O. C. (2007) Directed mutagenesis identifies amino acid residues involved in elongation factor Tu binding to yeast Phe-tRNA^{Phe}. *J. Mol. Biol. 368*, 119–130.
28. Roy, H., Becker, H. D., Mazauric, M. H., and Kern, D. (2007) Structural elements defining elongation factor Tu mediated suppression of codon ambiguity. *Nucleic Acids Res. 35*, 3420–3430.
29. Stanzel, M., Schon, A., and Sprinzl, M. (1994) Discrimination against misacylated tRNA by chloroplast elongation factor Tu. *Eur. J. Biochem. 219*, 435–439.
30. Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science 277*, 1453–1474.
31. Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C. (1997) The complete genome sequence of the gastric pathogen Helicobacter pylori. *Nature 388*, 539–547.
32. Cochella, L., and Green, R. (2005) An active role for tRNA in decoding beyond codon:anticodon pairing. *Science 308*, 1178–1180.
33. Ribeiro, S., Nock, S., and Sprinzl, M. (1995) Purification of aminoacyl-tRNA by affinity chromatography on immobilized *Thermus thermophilus* EF-Tu.GTP. *Anal. Biochem. 228*, 330–335.
34. Pleiss, J. A., and Uhlenbeck, O. C. (2001) Identification of thermodynamically relevant interactions between EF-Tu and backbone elements of tRNA. *J. Mol. Biol. 308*, 895–905.
35. Hunter, S. E., and Spremulli, L. L. (2004) Mutagenesis of glutamine 290 in *Escherichia coli* and mitochondrial elongation factor Tu affects interactions with mitochondrial aminoacyl-tRNAs and GTPase activity. *Biochemistry 43*, 6917–6927.
36. Louie, A., and Jurnak, F. (1985) Kinetic studies of *Escherichia coli* elongation factor Tu-guanosine 5′-triphosphate-aminoacyl-tRNA complexes. *Biochemistry 24*, 6433–6439.
37. Hunter, S. E., and Spremulli, L. L. (2004) Effects of mutagenesis of residue 221 on the properties of bacterial and mitochondrial elongation factor EF-Tu. *Biochim. Biophys. Acta 1699*, 173–182.
38. Pingoud, A., and Urbanke, C. (1980) Aminoacyl transfer ribonucleic acid binding site of the bacterial elongation factor Tu. *Biochemistry 19*, 2108–2112.
39. Min, B., Kitabatake, M., Polycarpo, C., Pelaschier, J., Raczniak, G., Ruan, B., Kobayashi, H., Namgoong, S., and Soll, D. (2003) Protein synthesis in *Escherichia coli* with mischarged tRNA. *J. Bacteriol. 185*, 3524–3526.
40. Shirvanee, L., Horn, V., and Yanofsky, C. (1990) *Escherichia coli* mutant trpA34 has an Asp----Asn change at active site residue 60 of the tryptophan synthetase alpha chain. *J. Biol. Chem. 265*, 6624–6625.
41. Nunez, H., Lefimil, C., Min, B., Soll, D., and Orellana, O. (2004) In vivo formation of glutamyl-tRNA(Gln) in *Escherichia coli* by heterologous glutamyl-tRNA synthetases. *FEBS Lett. 557*, 133–135.
42. Pingoud, A., Gast, F. U., Block, W., and Peters, F. (1983) The elongation factor Tu from *Escherichia coli*, aminoacyl-tRNA, and guanosine tetraphosphate form a ternary complex which is bound by programmed ribosomes. *J. Biol. Chem. 258*, 14200–14205.

BI800369Q