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Use of EF-Tu mutants for determining and improving aminoacylation efficiency and for purifying aminoacyl tRNAs with non-natural amino acids

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We present three methods relating to tRNA aminoacylation with non-natural amino acids using an *Escherichia coli* EF-Tu E215A/D216A mutant that can bind tightly to aa-tRNAs carrying either non-natural or natural amino acids: (i) a method for improving aminoacylation efficiency, (ii) a rapid method for analysing aminoacylation efficiency without the use of radioisotope labelling and (iii) a method for purifying aminoacyl-tRNAs. Although the EF-Tu mutant may be incompatible with some kinds of non-natural amino acids, we confirmed that the EF-Tu mutant could efficiently bind to aa-tRNAs carrying various amino acids (Arg, Ser, *O*-methyltyrosine, Bodipy FL-aminophenylalanine and 2-acrydonylalanine). These methods may be used for the efficient *in vitro* synthesis of proteins containing various non-natural amino acids.

Keywords: Aminoacyl RNA Synthesis/Biosynthesis/Elongation Factors/Ribonuclear Protein RNP/Transfer RNA/Translation.

Abbreviations: 2acdAla, 2-acrydonylalanine; 4azidoPhe, 4-azidophenylalanine; aa-tRNA, aminoacyl-tRNA; aaRS, aminoacyl-tRNA synthetase; acid PAGE, acid polyacrylamide gel electrophoresis; BFLAF, Bodipy FL-aminophenylalanine; EF-Tu, elongation factor Tu; meTyr, *O*-methyltyrosine.

Various *in vitro* methods have been developed to incorporate non-natural amino acids into proteins via engineered translation systems (*1*–*4*). In these methods, a tRNA corresponding to a non-natural amino acid must be pre-charged with the non-natural amino acid before the protein synthesis step, unless an orthogonal aminoacyl-tRNA synthetase (aaRS)–tRNA pair (*5*) is used. Typically, 100 pmol of a non-natural aminoacyl-tRNA is used in a 10-μl translation reaction mixture (*Escherichia coli* S30 system), which generates ~6 pmol of streptavidin protein containing the non-natural amino acid (*6*). However, the following problems arise when the tRNA is charged with various non-natural amino acids *in vitro*: (i) difficulty in quantifying aminoacylation efficiency with non-natural amino acids and (ii) low-charging efficiency with some non-natural amino acids.

Regarding the difficulty with quantification, the traditional, and most common, method for quantifying aminoacyl-tRNAs (aa-tRNAs) is the filter assay method, which uses radioisotope (RI)-labelled amino acids (*7*, *8*); however, RI-labelled amino acids are not available for most non-natural amino acids. Acid polyacrylamide gel electrophoresis (acid PAGE) is another method commonly used to analyse aminoacylation efficiency (*9*, *10*) because aa-tRNAs migrate slightly more slowly than free tRNAs in acid PAGE. However, depending on the nature of the amino acid, the aa-tRNA frequently fails to separate from the free tRNA. Thus, there is a need to develop a general method for analysing tRNA aminoacylation efficiency that can be used to quantify aminoacylation efficiency with non-natural amino acids.

Regarding the low charging efficiency, many non-natural amino acids can be attached to tRNAs using wild-type (*11*) or engineered (*2*, *5*) aaRSs; however, the tRNAs are not always aminoacylated efficiently with non-natural amino acids, especially when the tRNAs are mischarged with non-natural amino acids using wild-type aaRSs. Thus, a method for improving the aminoacylation efficiency with such non-natural amino acids would be helpful. In addition, a purification method that removes free tRNAs from aa-tRNAs is needed for when the aminoacylation efficiency is low.

In this study, we developed three methods using elongation factor Tu (EF-Tu) mutants to support aminoacylation with non-natural amino acids. First, we designed a method for raising aminoacylation efficiency; second, we devised a simple and fast method for analysing aminoacylation efficiency; and third, we developed a method for purifying aa-tRNAs (Fig. 1). EF-Tu is a protein that delivers aa-tRNAs to ribosomes in the translation system (*12*). We previously created *E. coli* EF-Tu mutants (*13*) that strongly bind to aa-tRNAs carrying either large non-natural amino acids or proteinogenic amino acids. In the current study, we used these EF-Tu mutants to optimize the preparation of aa-tRNAs that carry non-natural amino acids.

Materials and Methods

Buffers

Buffer A contained 50 mM HEPES-KOH (pH 7.6), 150 mM $(NH_4)_2SO_4$, 7 mM $MgCl_2$, 20% glycerol, 7 mM β-mercaptoethanol,

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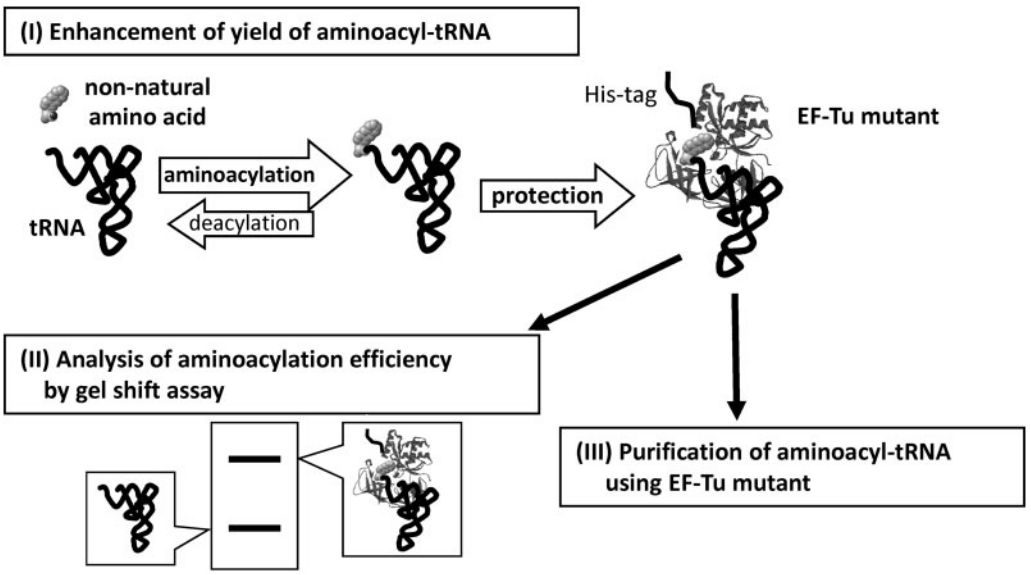

Fig. 1 Schematic diagram of the three objectives of this study. The aminoacylation reaction in the presence of the EF-Tu mutant (I) can readily be followed by analysis and purification (II and III, respectively) of the aminoacyl-tRNA.

100 μM phenylmethylsulfonyl fluoride and 15 μM guanosine diphosphate (GDP). Buffer B contained 50 mM HEPES–KOH (pH 7.6), 1 M NH_4Cl, 10 mM imidazole, 20% glycerol, 5 mM β-mercaptoethanol and 1 μM GDP. Buffer C consisted of 50 mM HEPES–KOH (pH 7.6), 100 mM $(NH_4)_2SO_4$, 150 mM imidazole, 20% glycerol, 5 mM β-mercaptoethanol and 2 μM GDP. Buffer D contained 50 mM HEPES–KOH (pH 7.6), 50 mM KCl, 10% glycerol, 1 mM dithiothreitol and 2 μM GDP. Buffers E and F were similar to buffer D, except that the concentrations of KCl were 0 and 300 mM, respectively. TC buffer contained 50 mM HEPES–KOH (pH 7.6), 65 mM NH_4OAc and 10 mM $Mg(OAc)_2$. AG loading solution contained 0.05% bromophenol blue, 0.05% xylene cyanol, 100 mM NaOAc (pH 5.0), 10 mM EDTA and 7 M urea. GSA loading solution contained 0.05% bromophenol blue and 50% glycerol. Elu buffer included 0.3 M KOAc (pH 4.5) and 7 M urea.

Preparation of EF-Tu mutants

Escherichia coli strain BL21(DE3) (Takara, Japan) was transformed with expression vectors encoding the His-tagged *E. coli* EF-Tu mutants (E215A, D216A and E215A/D216A), and grown and harvested as previously described (*13*). All purification procedures described below were performed at 4°C. Cellular pellets (~7 g) were resuspended in 7 ml of buffer A and lysed by sonication. The paste was then centrifuged for 30 min at 20,000*g* and the supernatant was collected and applied to a Ni–sepharose 6 Fast Flow column (250 μl; GE Healthcare, USA) equilibrated with buffer A. The column was washed with 30 ml of buffer A and then with 50 ml of buffer B. The His-tagged EF-Tu mutant was eluted from the column with buffer C. The fraction that contained the His-tagged EF-Tu mutant was dialysed against 500 ml of buffer D for 4 h with two buffer changes. For the complete removal of endogenous RNAs from the EF-Tu mutants, we employed the following procedures, which are different from those in our previous report (*13*). The dialysed fraction was concentrated to 1 ml on an Amicon Ultra-4 centrifugal filter device (MWCO = 10,000) (Millipore, USA), mixed with 250 μl of 5 mM ethylene diamine tetraacetic acid (EDTA), and incubated at 37°C for 30 min. The EDTA-treated fraction was loaded onto an Econo-Pac High Q Cartridge (1 ml; Bio-Rad, USA) equilibrated with buffer E, and the column was then washed with 2 ml of buffer E. The His-tagged EF-Tu mutant was eluted with 3 ml of buffer F, and buffer-exchange for buffer D was performed using an Amicon Ultra-4 centrifugal filter device. To prepare EF-Tu mutants that lacked the His-tag, thrombin treatment of the His-tagged protein was performed as described earlier (*14*). The protein concentration was estimated using a Protein Assay Kit (Bio-Rad). BSA was used as the standard.

Preparation of BFLAF-tRNA and 2acdAla-tRNA by chemical aminoacylation

Bodipy FL-aminophenylalanyl (BFLAF)-tRNA and 2-acrydonylalanyl (2acdAla)-tRNA were prepared by ligating transcribed tRNA without a 3′-CA dinucleotide unit and an aminoacylated dinucleotide (pdCpA) with T4 RNA ligase, as dpreviously earlier (*6*, *15*, *16*). The yeast $tRNA^{Phe}$ mutant bearing an amber anticodon ($tRNA^{Phe}_{CUA}$) and the *Methanosircina acetivorans* $tRNA^{Pyl}$ mutant bearing a CCCG four-base anticodon ($tRNA^{Pyl}_{CCCG}$) (*17*) were used as carriers for BFLAF and 2acdAla, respectively. Aa-tRNAs prepared by chemical aminoacylation were purified by phenol extraction and ethanol precipitation.

Preparation of aa-tRNAs using aaRSs

Yeast cytosolic $tRNA^{Arg2}$, a bovine mitochondrial $tRNA^{Ser}$ mutant bearing a CCCG four-base anticodon ($tRNA^{Ser}_{CCCG}$) (*17*) and a *Thermotoga maritima* $tRNA^{Tyr}$ mutant bearing an amber anticodon ($tRNA^{Tyr}_{CUA}$) were prepared by *in vitro* transcription, as described earlier (*17*). The tRNA transcripts were purified in a 10% denaturing polyacrylamide gel.

Yeast arginyl-tRNA synthetase (ArgRS) was prepared using an ArgRS-expression vector, which was kindly provided by Prof. Gilbert Eriani (CNRS, France) (*18*, *19*). *Thermotoga maritima* tyrosyl-tRNA synthetase (TyrRS) was expressed in *E. coli* BL21(DE3) using an expression vector that was prepared using pET-28b (Merck, Germany) and the TyrRS gene (accession No. AE000512-469) amplified by PCR from the *T. maritima* genome. Bovine mitochondrial seryl-tRNA synthetase (SerRS) was prepared as described (*20*). All these wild-type aaRSs were purified by Ni-NTA column chromatography as described earlier (*20*).

Yeast cytosolic $tRNA^{Arg2}$ was arginylated at 30°C for 20 min in a reaction mixture containing 50 mM HEPES–KOH (pH 7.6), 15 mM $MgCl_2$, 10 mM ATP, 30 mM KCl, 1 mM DTT, 30 μM [^{14}C]arginine (9.3 Bq/pmol, 361 cpm/pmol), 5 μM tRNA and 0.7 μM yeast ArgRS. Bovine mitochondrial $tRNA^{Ser}_{CCCG}$ was serylated at 37°C for 30 min in a reaction mixture containing 100 mM HEPES–KOH (pH 7.6), 10 mM $MgCl_2$, 20 mM KCl, 2 mM ATP, 1 mM DTT, 1 mM spermine, 1 mM serine, 5 μM tRNA and 0.7 μM bovine mitochondrial SerRS. *Thermotoga maritima* $tRNA^{Tyr}_{CUA}$ was aminoacylated at 50°C for 30 min in a reaction mixture containing 50 mM HEPES–KOH (pH 7.6), 10 mM $MgCl_2$, 2.5 mM ATP, 20 mM KCl, 2 mM DTT, 1 mM spermine, 500 μM tyrosine analogue, 5 μM tRNA and 0.5 μM *T. maritima* TyrRS.

Acid PAGE

Solutions containing aa-tRNA and/or tRNA were mixed with equal volumes of AG loading solution (10 mM EDTA and 7 M urea). Samples were separated by 7.5% PAGE at 4°C and 500 V

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for 18 h in a buffer containing 100 mM NaOAc (pH 5.0) and 10 mM EDTA. Gels were stained with SYBR Gold (Invitrogen, USA). Fluorescence images of SYBR Gold-stained gels ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 545$–565 nm) were obtained using an FMBIO III-SC01 imaging system (Hitachi, Japan). Evaluation of the band intensities and background subtraction were performed using ImageJ Ver.1.34s (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/), in accordance with the instructions in the software manual (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gel). Acid PAGE experiments were repeated three times, and the relative band intensities given in Figs 3, 4 and 6 are the averages of these three measurements.

Gel shift assay using EF-Tu mutants

The ternary complex of the EF-Tu mutant, GTP and aa-tRNA was prepared as follows. The EF-Tu mutant (40 pmol) was pre-incubated at 37°C for 15 min in 5 µl pre-incubation mixture containing 1 mM GTP, 40 mM HEPES–KOH (pH 7.6), 52 mM NH_4OAc, 8 mM $Mg(OAc)_2$, 16 mM phosphoenolpyruvate and 0.08 U/µl pyruvate kinase. Then, 20 pmol of aa-tRNA [dissolved in 3 µl of 1 mM KOAc (pH 5.0) or aminoacylation reaction solution] and 2 µl of aaBind buffer [150 mM HEPES-KOH (pH 7.6), 195 mM NH_4OAc and 30 mM $Mg(OAc)_2$] were added to the pre-incubated EF-Tu solution. The ternary complex mixture was incubated at 37°C for 2 min, then 2 µl of GSA loading solution was added to the mixture. Electrophoresis of samples was performed using 8 or 4% polyacrylamide gels at 4°C, in a buffer containing 50 mM Tris–HCl (pH 6.8), 65 mM NH_4OAc and 10 mM $Mg(OAc)_2$. Gels were stained with SYBR Gold or ethidium bromide. Evaluation of the band intensities and background subtraction were performed using ImageJ Ver.1.34s as described earlier. All gel shift assays were repeated three times, and the relative band intensities given in Figs 2, 3, 5 and 6 are the averages of these three measurements. Aminoacylation efficiencies [aa-tRNA(%)] were calculated from SYBR Gold- or ethidium bromide-stained gels according to the formula aa-tRNA(%) = (upper band intensity)/[(lower band intensity) + (upper band intensity)] × 100.

The image of [^{14}C]Arg-tRNA, and fluorescence images of 2acdAla and BFLAF were obtained without RNA staining. The image of [^{14}C]Arg-tRNA was obtained using a BAS-1000 (FUJIFILM, Japan). An imaging plate BAS-MS2040 (FUJIFILM) was exposed to the gel for 16 h. Fluorescence images of 2acdAla ($\lambda_{ex} = 405$ nm;

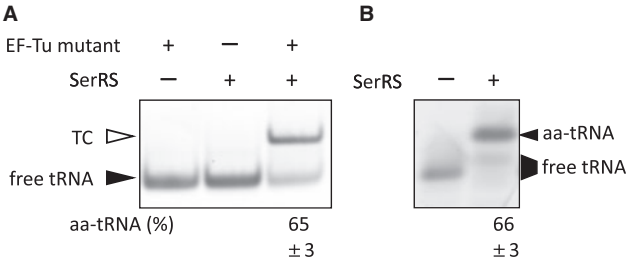

Fig. 3 Evaluation of methods used to estimate aminoacylation efficiency in the presence of the EF-Tu E215A/D216A mutant. A gel shift assay (A) and acid PAGE (B) were performed. Bovine mitochondrial $tRNA^{Ser}_{CCCG}$ was (+) or was not (−) serylated using bovine mitochondrial SerRS, and a reaction mixture containing 20 pmol of the tRNA was (+) or was not (−) mixed with the pre-incubated EF-Tu solution and loaded into each lane of (A). The open arrowhead indicates ternary complexes (TC) of the EF-Tu mutant, GTP and Ser-tRNA. The reaction mixture containing 20 pmol of tRNA and serylated (+) or non-serylated (−) bovine mitochondrial $tRNA^{Ser}_{CCCG}$ was loaded onto each lane of (B). Both gels were stained with SYBR Gold. The aminoacylation efficiency estimated from each gel is indicated below the gels (mean ± SD of three measurements).

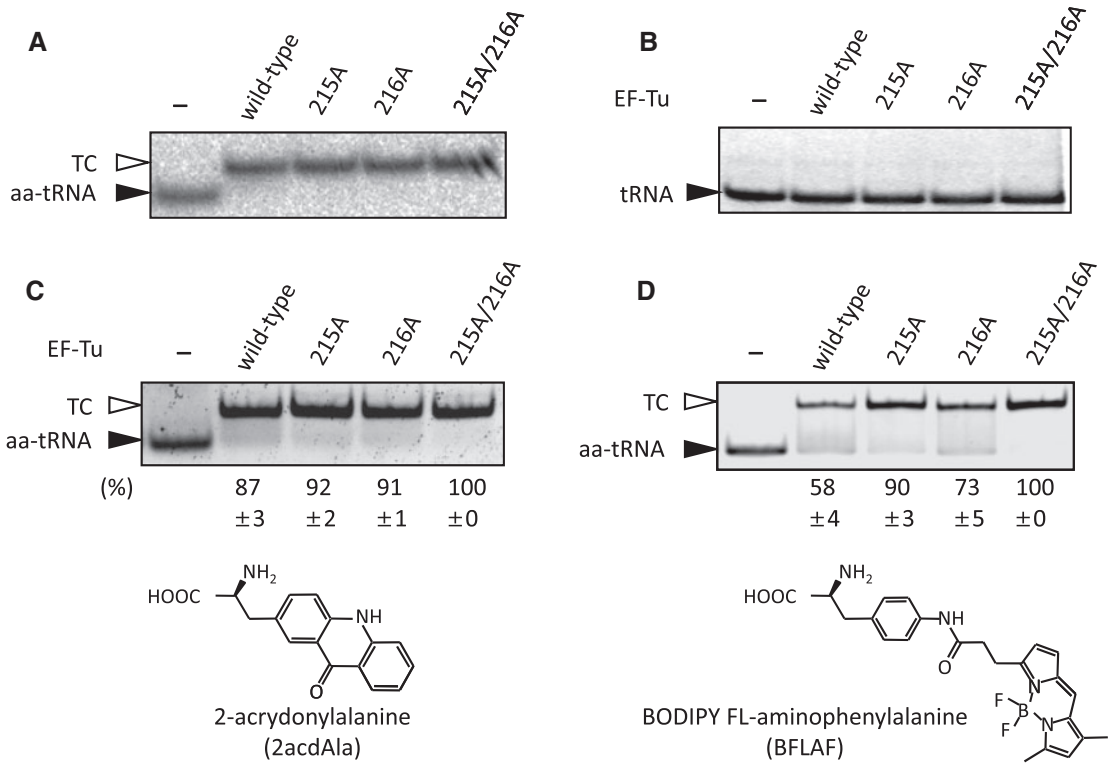

Fig. 2 EF-Tu variants bound exclusively to aminoacyl-tRNAs. Gel shift analysis of the ability of *E. coli* EF-Tu mutants (40 pmol) to bind to 20 pmol of [^{14}C]Arg-tRNA (A), free tRNA (B), 2acdAla-tRNA (C) and BFLAF-tRNA (D). Ternary complexes (TC) of EF-Tu mutants, GTP and aa-tRNAs are indicated by open arrowheads. (A) [^{14}C]Arg-$tRNA^{Arg2}$ prepared as described in the 'Materials and Methods' section was purified on a CentriSep spin column (Princeton Separations, USA), mixed with each EF-Tu mutant, and loaded onto the gel. The image of [^{14}C]Arg-tRNA was obtained using a BAS-1000 (FUJIFILM, Japan). (B) *Methanosircina acetivorans* $tRNA^{Pyl}_{CCCG}$ mixed with each EF-Tu mutant was analysed. The gel was stained with ethidium bromide. (C and D) Fluorescence images of 2acdAla and BFLAF were obtained using an FMBIO III-SC01. Binding efficiencies of 2acdAla-tRNA and BFLAF-tRNA to the EF-Tu variants (mean ± SD of three experiments) and the chemical structures of 2acdAla and BFLAF are shown below panels (C) and (D), respectively.

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λ_{em} = 450–470 nm) and BFLAF (λ_{ex} = 488 nm; λ_{em} = 545–565 nm) were obtained using an FMBIO III-SC01.

Purification of aa-tRNAs using the EF-Tu mutant

EF-Tu (240 pmol) was pre-incubated at 37°C for 15 min in 60 µl of preincubation mixture, as described earlier. Then, 36 µl of aminoacylation reaction mixture (including 120 pmol of aa-tRNA) and 24 µl of aaBind buffer were added to the mixture. The ternary complex mixture was incubated at 37°C for 2 min. After incubation, 5 µl of the mixture (the 'after incubation' fraction) was stored for PAGE analysis. A suspension of 5% Ni–NTA Magnetic Agarose Beads (50 µl; QIAGEN) was pipetted into a 1.5-ml tube, and the beads were washed with 500 µl of TC buffer. All of the following procedures were performed at 4°C. The 'after incubation' fraction (100 µl) was added to the beads and mixed gently using a Micromixer E-36 (TAITEC, Japan) for 30 min. The tube was placed on a magnetic separator (Qiagen 12-Tube Magnet) for 1 min, and the supernatant (*i.e.* the 'unbound' fraction) was removed. The beads in the tube were washed with 100 µl of TC buffer (creating the 'wash' fraction). One hundred microlitres of Elu buffer was added to the beads, which were then gently mixed for 5 min. The tube was placed on the magnetic separator for 1 min, and the supernatant (*i.e.* the 'elution' fraction) was collected. The aa-tRNA was collected from the elution fraction by phenol extraction and ethanol precipitation.

Quantification and purification of aa-tRNA following aminoacylation in the presence of the EF-Tu mutant

Thermotoga maritima $tRNA^{Tyr}_{CUA}$ was aminoacylated with *O*-methyltyrosine (meTyr) at 50°C for 30 min in 70 µl of the reaction mixture containing 50 mM HEPES–KOH (pH 7.6), 10 mM $MgCl_2$, 2.5 mM ATP, 20 mM KCl, 2 mM DTT, 1 mM spermine, 500 µM meTyr, 0.5 µM *T. maritima* TyrRS, 5 µM tRNA, 10 µM EF-Tu E215A/D216A mutant and 500 µM GTP. Four-microlitre aliquots of the reaction mixture, which included 20 pmol tRNA (meTyr-tRNA or free tRNA) and 40 pmol EF-Tu mutant, were directly mixed with 1 µl of GSA loading solution and analysed by gel shift assay, as described earlier, in 4% polyacrylamide gels. Purification of meTyr-tRNA from 50 µl of the aminoacylation reaction mixture was performed as follows. A 5% Ni–NTA magnetic bead suspension (30 µl) was washed with 300 µl of TC buffer in a microtube, and 50 µl of the aminoacylation reaction mixture was added to the beads. The slurry was gently mixed for 30 min. The tube was placed on a magnetic separator for 1 min, and the supernatant was removed. The beads in the tube were washed with 50 µl of TC buffer. Fifty microlitres of Elu buffer was added to the beads, which were then gently mixed for 15 min. The tube was placed on the magnetic separator for 1 min and the supernatant was collected.

Results

Estimation of aminoacylation efficiency using EF-Tu mutants

In order to measure aminoacylation efficiency by gel shift assay using EF-Tu or its mutants, the EF-Tu protein must bind quantitatively to various aa-tRNAs, but must not bind at all to free tRNAs in the gel. Therefore, we evaluated the binding efficiencies of various aa-tRNAs for wild-type and mutant (E215A, D216A and E215A/D216A) *E. coli* EF-Tu by gel shift assay. First, we confirmed that Arg-tRNA bound quantitatively (>99%) to all of the EF-Tu variants (Fig. 2A) when EF-Tu at twice the molar equivalent of aa-tRNA was used. We also confirmed that all of the EF-Tu variants did not bind to free tRNA (Fig. 2B). Wild-type EF-Tu binds efficiently to aa-tRNAs carrying proteinogenic amino acids, but not to aa-tRNAs carrying certain types of non-natural amino acids (*13*). We therefore evaluated the binding efficiencies of EF-Tu variants for 2acdAla-tRNA and BFLAF-tRNA (Fig. 2C and D), and established that the order of binding efficiency (from strongest to weakest) was the E215A/D216A mutant, the E215A mutant, the D216A mutant and the wild-type. Although wild-type EF-Tu did not bind quantitatively to these aa-tRNAs carrying non-natural amino acids, the E215A/D216A mutant did bind them quantitatively. Thus, the E215A/D216A mutant of EF-Tu does not bind to free tRNA, but does bind quantitatively to aa-tRNAs carrying natural or non-natural amino acids.

Next, we examined whether aminoacylation efficiency could be quantified by a gel shift assay using the EF-Tu E215A/D216A mutant. As a model experiment, we serylated $tRNA^{Ser}_{CCCG}$ using bovine mitochondrial SerRS and analysed it with both a gel shift assay and acid PAGE (Fig. 3). All aa-tRNAs formed ternary EF-Tu/GTP/aa-tRNA complexes (the upper-shifted band, indicated by 'TC' in Figs 2 and 3A), and all free tRNAs were included in the lower band in the gel shift assay. The free tRNA band in Fig. 3B is broad because the $tRNA^{Ser}_{CCCG}$ fraction also included tRNAs that were 1-nt longer or shorter than the $tRNA^{Ser}_{CCCG}$, which are byproducts of T7 transcription (*21*) and could not be excluded by the tRNA purification procedure described earlier. The estimation of aminoacylation efficiency by gel shift assay (Fig. 3A) was very close to that estimated by acid PAGE (Fig. 3B). We additionally aminoacylated yeast $tRNA^{Arg2}$ with [^{14}C]Arg as described earlier and measured the aminoacylation efficiency with a gel shift assay as well as with a conventional filter assay performed in accordance with (*20*). The efficiencies estimated by the gel shift and filter assays were 37 ± 1 and 36 ± 1%, respectively. These results indicate that tRNA aminoacylation efficiency can be quantified with gel shift assays using the EF-Tu mutant.

Purification of aa-tRNAs using the EF-Tu mutant

A purification method to exclude free tRNAs from aa-tRNA is needed when aminoacylation efficiency is low. We examined whether aa-tRNA could be purified from His-tagged EF-Tu variants (wild-type EF-Tu and the E215A/D216A mutant). A mixture of 2acdAla-tRNA and unacylated tRNA was used for evaluating the purification efficiency and aa-tRNA recovery. 2acdAla-tRNA was prepared as described in the 'Materials and Methods' section, and unacylated tRNAs were included in the preparation. The aminoacylation efficiency was 46%. The aminoacylated (46%) and unacylated (54%) tRNA mixture was mixed with the EF-Tu variant and then with Ni–NTA magnetic beads. The beads were washed with TC buffer and then eluted with Elu buffer. Fig. 4A shows that 2acdAla-tRNA could be purified using wild-type or mutant EF-Tu proteins, and free tRNA was completely excluded (see 'elution' lanes). Although EF-Tu variants were also included in the 'elution' fraction (Fig. 4B), they can be removed by phenol extraction. The recovery efficiency of 2acdAla-tRNA using the E215A/D216A mutant (70%) was markedly higher than that using wild-type EF-Tu (42%).

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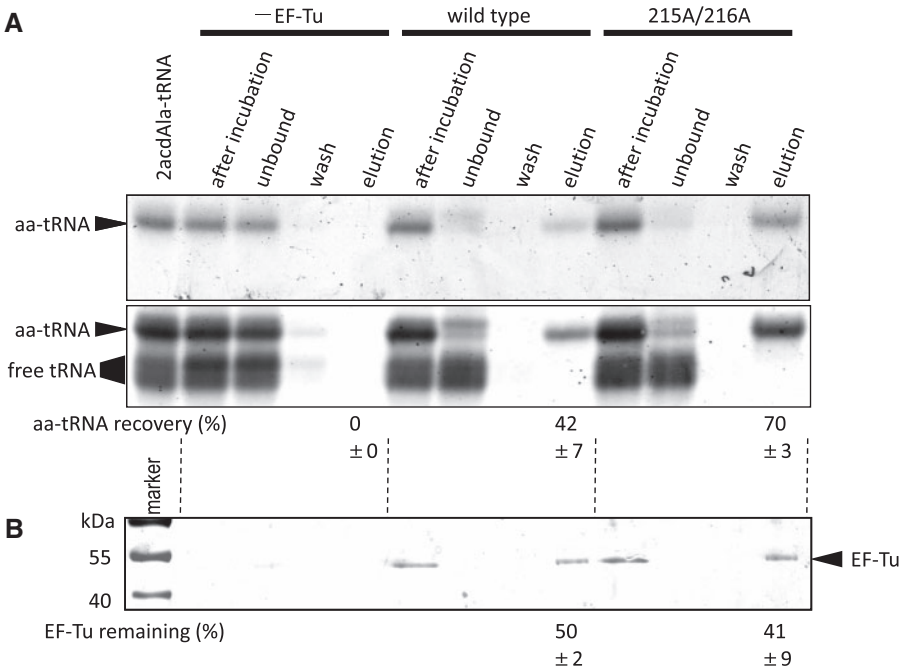

Fig. 4 Purification of 2acdAla-tRNA using wild-type or mutant EF-Tu. Fractions obtained during various purification steps of 2acdAla-tRNA using the E215A/D216A mutant and Ni^{2+} magnetic beads were analysed by acid PAGE (A) and sodium dodecyl sulphate (SDS)–PAGE (B). (A) Each fraction (5 μl) was mixed with 5 μl of AG loading solution and applied to the gel. The upper panel shows the 2acdAla fluorescence image ($\lambda_{ex} = 405$ nm; $\lambda_{em} = 450$–470 nm) before staining of the gel. The lower panel is the SYBR Gold-stained image. Aminoacyl-tRNA recovery is indicated below each lane containing 'elution' fractions. (B) The same samples as in A were analysed by 10% SDS–PAGE. The gel was stained with Coomassie Brilliant Blue.

Aminoacylation in the presence of EF-Tu mutants

tRNAs are not always efficiently aminoacylated with non-natural amino acids when wild-type or engineered aaRSs are used. For example, the aminoacylation efficiencies of $tRNA^{Tyr}_{CUA}$ with *O*-methyltyrosine (meTyr) and 4-azidophenylalanine (4azidoPhe) using *T. maritima* TyrRS were both 14% (Fig. 5). In an attempt to increase the aa-tRNA yield, we added EF-Tu variants (10 μM) and GTP (500 μM) to the aminoacylation reaction mixture during the reaction. We found that the yields of tRNAs charged with meTyr and 4azidoPhe were in the following order: the E215A/D216A mutant ≈ the E215A mutant > the D216A mutant > wild-type EF-Tu (Fig. 5). The reproducibility of this order was confirmed by repeating these experiments three times.

Quantification and purification of aa-tRNA following aminoacylation in the presence of the EF-Tu mutant

In the aa-tRNA quantification and purification experiments (Figs 3 and 4), EF-Tu variants were added to aa-tRNAs that had been prepared beforehand. In the current experiment, an aa-tRNA was prepared in the presence of the E215A/D216A mutant and then quantified and purified. Aminoacylation of tRNA in the presence of the EF-Tu mutant increased aa-tRNA yield (Fig. 5), and the resultant reaction mixture could be directly used for the EF-Tu-based quantification and purification methods (Fig. 6). The *O*-methyltyrosylation efficiency of the $tRNA^{Tyr}_{CUA}$ obtained with a gel shift assay (41%) was almost the same as the efficiency with acid PAGE (43%; Fig. 6). This experiment was repeated three times, and the aminoacylation efficiencies estimated by gel shift assay always agreed with those by acid PAGE. meTyr-tRNA was efficiently recovered (74%) by the purification procedure, and free tRNAs were not present in the elution fraction (Fig. 6).

Discussion

When *in vitro* methods are used to incorporate non-natural amino acids into proteins, a tRNA corresponding to a non-natural amino acid must be precharged with the non-natural amino acid prior to the protein synthesis step, unless an orthogonal aaRS-tRNA pair is used (*1*–*4*, *6*, *11*, *22*). In this study, we developed the following three methods to support aminoacylation with non-natural amino acids using the EF-Tu mutant, which can tightly bind to aa-tRNAs carrying non-natural amino acids (*13*): (i) a method to raise aminoacylation efficiency, (ii) a simple method to analyse aminoacylation efficiency without RI-labelling, and (iii) a method to purify the aa-tRNAs.

We confirmed that the EF-Tu E215A/D216A mutant bound efficiently to aa-tRNAs carrying natural amino acids (Ser and Arg) as well as to non-natural amino acids (meTyr, 2acdAla, BFLAF); however, the EF-Tu mutant should be tested for its binding to a wider range amino acids in the future. There was no difficulty in preparing the EF-Tu mutant, which was obtained using the same expression and purification procedures as for wild-type EF-Tu. We did not observe

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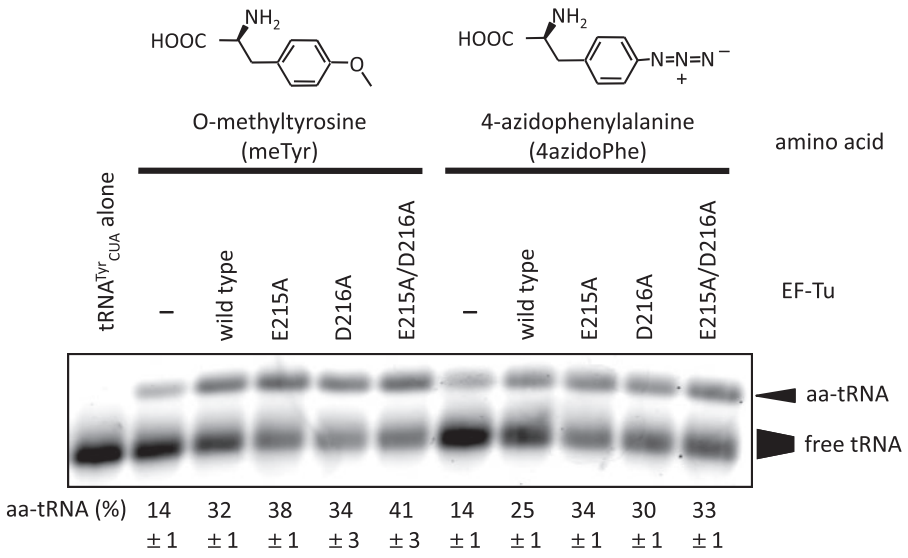

Fig. 5 Improvement of aminoacylation yield using EF-Tu mutants. Acid PAGE was performed to analyse *T. maritima* $tRNA^{Tyr}_{CUA}$ aminoacylated with non-natural amino acids in the absence (−) or presence of the indicated EF-Tu variants. Aminoacylation reaction mixtures containing 20 pmol aa-tRNA or tRNA were directly mixed with AG loading solution and applied to the gel. The aminoacylation efficiencies are shown beneath the gel (means ± SD of three gels).

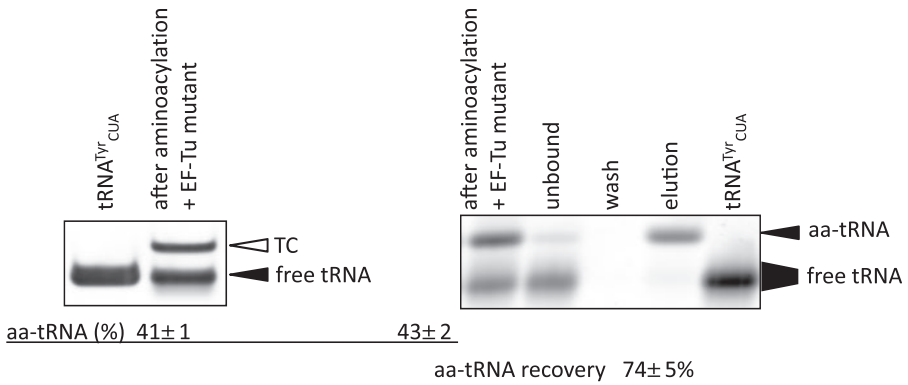

Fig. 6 Quantification and purification of meTyr-tRNA following aminoacylation in the presence of the EF-Tu mutant. The aminoacylation reaction mixture containing the EF-Tu E215A/D216A mutant was directly mixed with 1 µl of GSA loading solution and loaded onto the gel for a gel shift assay (left panel). Ternary complexes (TC) of EF-Tu mutants, GTP and aa-tRNAs are indicated by the open arrowhead. The aminoacylation reaction mixture was purified using Ni^{2+} magnetic beads, and 4 µl of the fraction obtained at each purification step was phenol extracted, ethanol precipitated, redissolved in 4 µl of 1 mM KOAc (pH 4.5) and 4 µl of AG loading solution, and analysed by acid PAGE (right panel).

a cross-species barrier between *E. coli* EF-Tu and tRNAs from other species (yeast, bovine mitochondria, *M. acetivorans* and *T. maritima*). These findings suggest that the EF-Tu mutant is an efficient tool for tRNA aminoacylation, especially for aminoacylation with non-natural amino acids that are incompatible with wild-type EF-Tu.

It should be noted that the gel shift assay using the EF-Tu mutant was much faster to perform than the acid PAGE analysis, which is commonly used to analyse aminoacylation efficiency. The gel shift assay (Fig. 3A) was performed on a 7-cm long, 8% polyacrylamide gel at 100 V for 30 min, and the migration distances of the ternary complexes and free tRNAs were 1.0 and 2.0 cm, respectively. The gel shift assay presented in Fig. 6 was performed on a 7-cm-long, 4% polyacrylamide gel at 100 V for '15 min', and the migration distances of the ternary complexes and free tRNAs were 0.5 and 1.0 cm, respectively. In contrast, acid PAGE (Fig. 3B) was performed on 40-cm-long, 7.5% polyacrylamide gels at 500 V for '16 h', and the migration distances of Ser-tRNA and free tRNAs were 31.4 and 32.2 cm, respectively. Therefore, aa-tRNAs and free tRNAs were more rapidly separated by gel shift assay than by acid PAGE.

Although a wild-type EF-Tu can be used for the evaluation of aminoacylation efficiency (*23*), it cannot bind quantitatively to aa-tRNAs carrying bulky non-natural amino acids such as 2acdAla and BFLAF (Fig. 2C and D). Thus the use of the EF-Tu mutant is necessary for evaluation of efficiency of aminoacylation using non-natural amino acids. In addition to our method, a method reported by Wolfson *et al.* (*24*) that involves charging of a nicked tRNA and

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subsequent separation of an aminoacylated 3′-fragment may be suitable for the rapid analysis of the efficiency of aminoacylation performed using unlabelled non-natural amino acids. However, a pre-requisite for this method is that each nicked tRNA of interest must have the same activity as the intact tRNA.

Aminoacylation efficiencies with meTyr and 4azidoPhe increased in the presence of EF-Tu variants, especially the E215A/D216A and E215A mutants (Fig. 5). This could be because EF-Tu protects aa-tRNAs from deacylation (*25*) or because it possesses chaperone activity (*26*–*28*). The ability of EF-Tu variants to protect against deacylation is most likely the major reason for the increased aa-tRNA yield, as the degree to which each EF-Tu variant raises the aa-tRNA yield corresponds with its binding efficiency to various non-natural aa-tRNAs (Fig. 2C and D) (*13*). The *E. coli* EF-Tu/GDP complex is a significantly more active chaperone than is EF-Tu/GTP (*26*). In the experiment presented in Fig. 5, we added GTP to the EF-Tu variants. Compared to EF-Tu/GDP, EF-Tu/GTP significantly improved aminoacylation yield (data not shown), suggesting that the chaperone activity of EF-Tu variants made only a minor contribution, if any, to the increase in aminoacylation yield.

Both 2acdAla-tRNA and meTyr-tRNA were efficiently purified using the E215A/D216A mutant (Figs 3 and 6). Although EF-Tu-based aa-tRNA purification methods using resin-immobilized *E. coli* and *Thermus thermophilus* EF-Tus were previously reported (*29*, *30*), wild-type EF-Tu proteins were used in those studies. The aa-tRNA purification method using the E215A/D216A mutant would be more efficient than using wild-type EF-Tu for recovering purified aa-tRNAs carrying non-natural amino acids, especially large non-natural amino acids.

In conclusion, the aa-tRNA quantification method presented in this study enables the rapid evaluation of aminoacylation efficiencies with non-natural amino acids. For some non-natural amino acids that cannot be efficiently attached to tRNAs, the methods for improving aminoacylation efficiency and purification of aa-tRNAs presented here would be helpful for the preparation of larger amounts of pure aa-tRNAs.

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Conflict of interest

None declared.

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