

# Improving amber suppression activity of an orthogonal pair of *Saccharomyces cerevisiae* tyrosyl-tRNA synthetase and a variant of *E. coli* initiator tRNA, *fMam* tRNA<sub>CUA</sub>, for the efficient incorporation of unnatural amino acids<sup>§</sup>

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## 효율적인 비천연 아미노산 도입을 위한 효모균 타이로신-tRNA 합성효소와 대장균 시작 tRNA 변이체의 엠버서프레션 활성증가<sup>§</sup>

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The orthogonal pair of *Saccharomyces cerevisiae* tyrosyl-tRNA synthetase (*Sc* YRS) and a variant of *E. coli* initiator tRNA, *fMam* tRNA<sub>CUA</sub> which recognizes the amber stop codon is an effective tool for site-specific incorporation of unnatural amino acids into the protein in *E. coli*. To evolve the amber suppression activity of the orthogonal pair, we generated a mutant library of *Sc* YRS by randomizing two amino acids at 320 and 321 which involve recognition of the first base of anticodon in *fMam* tRNA<sub>CUA</sub>. Two positive clones are selected from the library screening with chloramphenicol resistance mediated by amber suppression. They showed growth resistance against high concentration of chloramphenicol and their IC<sub>50</sub> values were approximately 1.7~2.3 fold higher than the wild type YRS. *In vivo* amber suppression assay reveals that mutant YRS-3 (mYRS-3) clone containing amino acid substitutions of P320A and D321A showed 6.5-fold higher activity of amber suppression compared with the wild type. In addition, *in vitro* aminoacylation kinetics of mYRS-3 also showed approximately 7-fold higher activity

than the wild type, and the enhancement was mainly due to the increase of tRNA binding affinity. These results demonstrate that optimization of anticodon recognition by engineered aminoacyl tRNA synthetase improves the efficiency of unnatural amino acid incorporation in response to nonsense codon.

**Keywords:** amber suppression, aminoacyl-tRNA synthetase, anticodon recognition, genetic code expansion, unnatural amino acid

The development of a method that allows the cell to genetically encode additional amino acids other than 20 canonical amino acids might enable the evolution of proteins, or even entire organisms, with new or enhanced properties. Expanding genetic code of organisms via a pair of orthogonal aminoacyl-tRNA synthetase (aaRS) and its cognate tRNA is a widely used method, and so far, more than 167 unnatural amino acids (UAA) are being incorporated in diverse organisms such as bacteria, yeast, plant, and mammalian cells (Liu and Schultz, 2010; Chin, 2017; Mukai *et al.*, 2017). The key requirement to genetically encode UAAs is the design of novel aaRS/suppressor tRNA

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pair that can specifically incorporate UAA into protein in response to the nonsense triple codon or quadruplet codon (Liu *et al.*, 1997; Wang *et al.*, 2001; Anderson *et al.*, 2004). Moreover, the aaRS/tRNA pair must be ‘orthogonal’ to the host, meaning that the aaRS should not aminoacylate the endogenous tRNAs of the host and the tRNA should not be aminoacylated by the host endogenous aaRSs (Liu *et al.*, 1997; Liu and Schultz, 2010). Several orthogonal aaRS/tRNA pairs have been used for this purpose in *E. coli*, which include pairs derived from *Saccharomyces cerevisiae* (*Sc*) such as DRS/tRNA<sup>Asp</sup>, QRS/tRNA<sup>Gln</sup>, and YRS/tRNA<sup>Tyr</sup> (Liu *et al.*, 1997; Pastrnak *et al.*, 2000; Ohno *et al.*, 2001), a pair of *Methanocaldococcus jannaschii* (*Mj*) YRS/tRNA<sup>Tyr</sup> (Wang *et al.*, 2001), and a hybrid pair of *Sc* YRS/*fMam* tRNA<sub>CUA</sub>, a variant of *E. coli* initiator tRNA<sub>2<sup>fMet</sup></sub> (Kowal *et al.*, 2001).

For the site-specific incorporation of UAAs, orthogonal amber suppressor tRNAs are commonly used because amber codon is not only the least used stop codon (about 7% of total genes) but also rarely terminates essential genes in *E. coli* (Nakamura *et al.*, 2000). However, changes in base sequences of the anticodon of amber suppressor tRNAs deteriorate tRNA recognition activity of the orthogonal aaRS, which result in low yields of protein products containing desired UAAs. Thus, efforts towards optimization of suppressor tRNA recognition by its orthogonal aaRS pair are highly desirable to increase efficiency and fidelity of UAA incorporation. In this regard, studies for the crystal structure of a ternary complex of YRS/tRNA<sup>Tyr</sup>/tyrosine from *M. jannaschii* were very informative to evolve *Mj* YRS, which enables to enhance aminoacylation activity to the amber suppressor tRNA (Kobayashi *et al.*, 2003; Wang *et al.*, 2015).

*Sc* YRS and a variant of *E. coli* initiator tRNA<sub>2<sup>fMet</sup></sub> (*fMam* tRNA<sub>CUA</sub>) function as an effective orthogonal tool for site-specific incorporation of UAAs into protein in *E. coli* (Kowal *et al.*, 2001). However, *Sc* YRS which originally recognizes tyrosine anticodon GUA poorly aminoacylated *fMam* tRNA<sub>CUA</sub> because its anticodon was changed to CUA complementary to amber stop codon, and showed weak amber suppression activity (Edan *et al.*, 2014). Thus, to improve amber suppression efficiency of the orthogonal pair of *Sc* YRS and *fMam* tRNA<sub>CUA</sub>, here we applied the directed evolution approach to *Sc* YRS based on the x-ray crystal structure of *Sc* YRS-tRNA<sup>Tyr</sup>-tyrosine-AMP complex.

According to the crystal structure, the first anticodon G34 is flipped out from the back-bone of tRNA and specifically interacts with Pro320 and D321 residues of *Sc* YRS (Tsunoda *et al.*, 2007). Since the second and third anticodon bases, U35 and A36, are same to those of the cognate tRNA<sup>Tyr</sup>, and have fewer base-specific interactions with the synthetase, we excluded residues consisting of the other part of anticodon-binding pocket from the mutation targets. After screening a library generated by randomly mutating the two residues, P320 and D321, two mutant clones were selected and their aminoacylation activity was measured both *in vivo* and *in vitro*.

## Materials and Methods

### *E. coli* strains, culture media, and plasmids

*E. coli* strain DH10B [F<sup>+</sup>*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ* Δ*M15* Δ*lacX74* *deoR* *recA1* *araD139* Δ(*ara-leu*)7697 *galU* *galK* *rpsL* *nupG* *tonA*] (Invitrogen) and a genetically modified strain DH10B(Tn:*lacZam*), which contains an amber-mutated *lacZ* gene in the DH10B chromosome (Kim *et al.*, 2009), were used in this study. LB and GMML media supplemented with antibiotics (100 μg/ml ampicillin, 25 μg/ml tetracycline, and 0–600 μg/ml chloramphenicol), if necessary, were used for bacterial culture. Concentrated M9 salt solution (5X) was prepared by dissolving 30 g Na<sub>2</sub>HPO<sub>4</sub>, 15 g KH<sub>2</sub>PO<sub>4</sub>, 2.5 g NaCl, 5 g NH<sub>4</sub>Cl in 1 L deionized water. GMML medium was prepared by adding 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 0.4% glucose, 1% glycerol, 0.3 mM Leu to 1X M9 salt solution.

Chloramphenicol acetyltransferase (CAT) gene in the vector frame of pQE30 (Qiagen) was deleted by restriction digestion of *Pvu*II and *Nco*I and subsequent self-ligation. *Sc* YRS gene from pKK-YRS\*9 (Kowal *et al.*, 2001) was subcloned into CAT-deleted pQE30 by using restriction sites of *Bam*HI and *Pst*I, thereby constructing *Sc* YRS expression vector pQE-YRS. The pACamG encodes a CAT gene containing an amber mutation at the 27<sup>th</sup> residue and a suppressor tRNA derived from mutant *E. coli* initiator tRNA gene *trnfMT2:A71/T35A36/G72* (*fMam* tRNA<sub>CUA</sub>) (Chow and RajBhandary, 1993).

### Mutant library construction

For the random mutation of P320 and D321 residues of *Sc*

YRS, we performed PCRs composed of 2 steps. As shown in Fig. 1A, we carried out the first round PCR with degenerative primers encoding P320X and D321X amino acid residues. After combining purified PCR products of the first round PCR at equimolar ratio, the second round PCR was performed with P11 and P14 primers that contain *Bst*XI and *Pst*I restriction sites, respectively. The primer sequences used in PCR were as follows; P11; 5'-AGATGTTGATTGCCAATTTGGTGG-3', P12; 5'-GATAGCGTCAGCAACAATTTTTAGNNNNNN TGGGGACAATTTTC-3', P13; 5'-CTAAAAATTGGTGT TGCTGACGCTATC-3', P14; 5'-AAAACTGCAGTTACA ATTTGGTTTCCTCTAG-3'. After 30 cycles of PCR, each product was analyzed by electrophoresis on 1% agarose gel. The final PCR product was purified and cut with *Bst*XI & *Pst*I restriction enzymes and subcloned into pQE-YRS plasmid at the same restriction sites. The purified final PCR product was also directly sequenced to verify the randomized mutation.

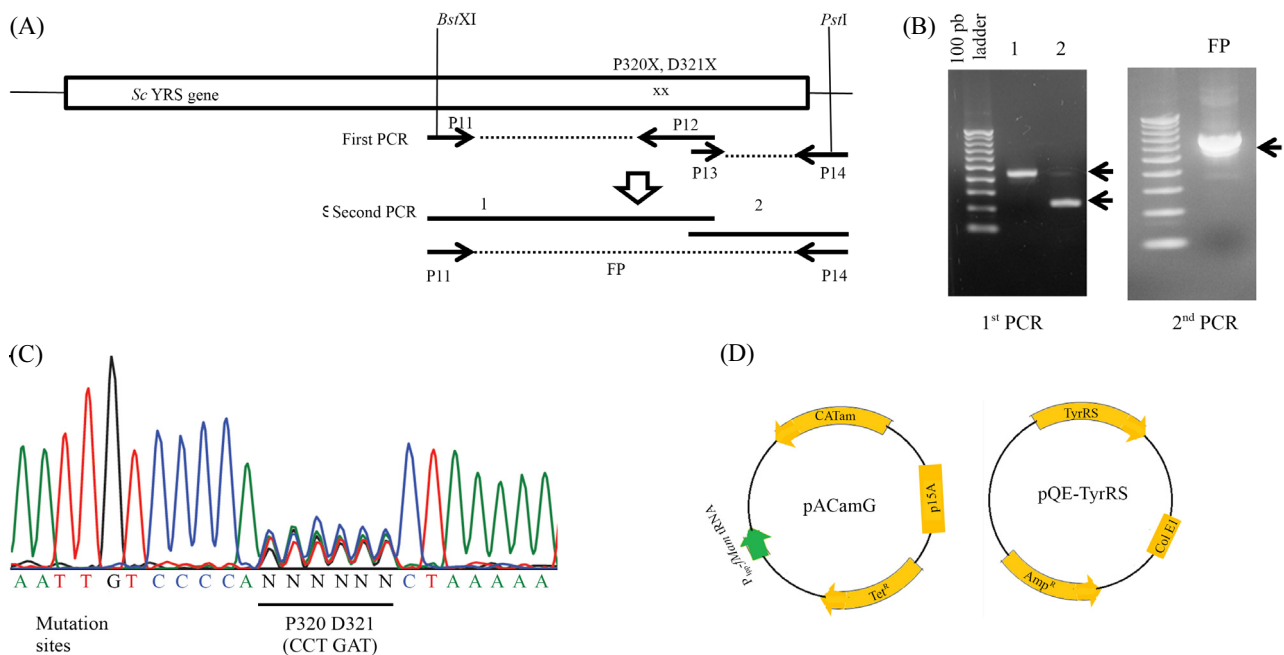
To measure the library titer, 1  $\mu$ l of ligation reaction was transformed to DH10B competent cells (Invitrogen) via electroporation (2.0 kv, 200  $\Omega$ , 25  $\mu$ F), rescued with SOC media for 40 min, and spread (100  $\mu$ l) on LB plates containing 100  $\mu$ g/ml ampicillin. After overnight culture, library titer was measured

by counting the growing colonies (CFU), and the library size was estimated at  $5.5 \times 10^3$  CFU/ $\mu$ l.

### Library screening

For library screening, 2  $\mu$ l of library construct was transformed into DH10B competent cells containing pACamG by electroporation. The transformants were allowed to grow on LB plates containing 100  $\mu$ g/ml ampicillin, 25  $\mu$ g/ml tetracycline, and different concentration of chloramphenicol such as 100, 200, and 300  $\mu$ g/ml for 16 h at 37°C.

Eight clones were isolated from the first screen on LB plates containing 300  $\mu$ g/ml of chloramphenicol and further analyzed for their chloramphenicol resistance. Same amount of seed culture of each clone was inoculated into LB broth medium and GMLL medium containing 100  $\mu$ g/ml ampicillin, 25  $\mu$ g/ml tetracycline, and increasing concentrations of chloramphenicol (0~1,000  $\mu$ g/ml). Growth rate was determined by measuring optical density of the bacterial cultures at 600 nm every 6 h. The clone containing pQE-YRS which expresses wild-type YRS (wt-YRS) was also tested as a control. Finally, two positive clones (mYRS-3 and mYRS-5) out of eight clones have been selected and their YRS DNA sequences were read in both



**Fig. 1. Generation of a random mutation library of *Sc* YRS at P320 and D321 residues.** (A) Strategy for library construction by PCR. P12 primer contains randomly degenerated sequences encoding P320 and D321 residues. (B) Agarose gel electrophoresis of PCR products. Both the first (left) and the second (right) PCR products migrate at the expected position. (C) Electropherogram of nucleotide sequence encompassing mutation sites from the final PCR product indicates randomized nucleotide sequence encoding P320 and D321 residues. (D) Diagram of plasmids used in this experiments.

directions to reveal amino acid substitution.

### $\beta$ -galactosidase assay for *in vivo* amber suppression activity

*E. coli* DH10B (Tn:*lacZam*), which contains amber-mutated *lacZ* gene in the genomic DNA (Kim *et al.*, 2009), was cotransformed with pACamG and plasmid expressing wt-YRS, mYRS-3, or mYRS-5. The transformant of each mutant was cultured overnight in 1 ml LB broth media containing 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml tetracycline. Next day, same volume of each seed culture (1:50) was inoculated into 2 ml of fresh LB broth media containing 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml tetracycline. After 4 h culture, 0.5 mM IPTG was added to the culture to induce YRS expression for 4 h. Bacterial culture (1.5 ml) was harvested and washed once with Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 50 mM 2-mercaptoethanol, pH7.0). Cell pellet was resuspended in 50  $\mu$ l of lysis buffer (1 mg/ml lysozyme, 30 mM Tris-Cl pH8.0, 1 mM EDTA, 20% Sucrose) and incubated for 4 min at room temperature. After adding 100  $\mu$ l of Z buffer, 15  $\mu$ l of 0.1% SDS, and 30  $\mu$ l of chloroform to the cell resuspension, cells were broken by vortexing for 10 sec. After centrifugation at 12,000 rpm for 10 min, the aqueous layer was taken for  $\beta$ -galactosidase assay. Enzyme reaction mixture consisting of 100  $\mu$ l of cell extract, 200  $\mu$ l of Z buffer, and 30  $\mu$ l of 5 mM 4-methylumbelliferyl- $\beta$ -D-galactopyranoside (MUG) was incubated for 30 min at 37°C. Reaction was stopped by adding 500  $\mu$ l of 1 M Na<sub>2</sub>CO<sub>3</sub> and fluorescence was measured at 360 nm excitation and 440 nm emission. In addition, protein quantitation was carried out with the same cell extracts by the Bradford method (Bio-Rad protein assay) and the protein concentration of each cell extract was used for normalization of  $\beta$ -galactosidase activity.

### Recombinant YRS purification

To test *in vitro* aminoacylation activity of mutant YRS, pQE-YRS expressing either wt-YRS or mYRS-3 was transformed into *E. coli* M15 strain. Each transformant was cultured in 200 ml LB containing 100  $\mu$ g/ml ampicillin and protein expression was induced by the treatment of 1 mM IPTG for 4 h. Since the recombinant YRS proteins were produced with an N-terminal 6xHis tag, they were purified by the immobilized metal ion affinity chromatography of HiPur cobalt resin (ThermoFisher

Scientific) at 4°C according to manufacturer's protocol. Briefly, instead of phosphate buffer, Tris-based buffer was used for protein purification to prevent contamination of inorganic phosphate in the *in vitro* aminoacylation reaction. The compositions of buffers were as follows; lysis buffer (50 mM Tris-HCl, 300 mM NaCl, and 10 mM 2-mercaptoethanol, pH 7.4), washing buffer (50 mM Tris-HCl, 300 mM NaCl, and 10 mM imidazole, pH 7.4), and elution buffer (50 mM Tris-HCl, 300 mM NaCl, and 150 mM imidazole, pH 7.4). Eluted protein was dialyzed in dialysis buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.4) with cut-off molecular size of 8 kDa. After dialysis, protein concentration was measured by the Bradford method (Bio-Rad protein assay).

### *In vitro* aminoacylation assay

*In vitro* aminoacylation assay was performed in aminoacylation buffer (30 mM HEPES, 140 mM NaCl, 30 mM KCl, and 40 mM MgCl<sub>2</sub>), 1 mM tyrosine, 1 mM DTT, 200  $\mu$ M ATP, and 200 U/ml inorganic pyrophosphatase (PPiase), 10  $\mu$ g/ml of wt-YRS or mYRS-3, and 0~25.8  $\mu$ M of *fMam* tRNA<sub>CUA</sub>. The aminoacylation reactions (30  $\mu$ l total reaction volume) were performed in clear, flat-bottom 96-well plates and incubated for 30 min at 37°C. Reactions were stopped by adding 20  $\mu$ l of 0.3 M EDTA and 100  $\mu$ l of malachite green reagent (Echelon Biosciences). After incubating for 30 min at room temperature for color development, absorbance was measured at 620 nm by using Infinite M200 Pro Spectrophotometer (Nano-Quant). Inorganic phosphate production was calculated from the standard curve produced according to manufacturer's protocol.

### Statistical analysis

The experiments were repeated at least more than 3 time to obtain reproducibility, the mean, and standard deviation. For statistical analysis, Student's unpaired *t*-test was performed.

## Results and Discussion

### Generation of mutant library of *Sc* YRS randomizing the amino acid residues at 320 and 321

For directed evolution of amber suppression activity of the orthogonal pair of *Sc* YRS and *fMam* tRNA<sub>CUA</sub>, we generated

a mutant library of YRS by randomizing the nucleotide sequence encoding two amino acid residues, P320 and D321, which are known to be involved in recognition of the first anticodon base G34 in X-ray crystallography (Tsunoda *et al.*, 2007). A degenerated primer was used to randomize the mutation sites and two rounds of PCR were carried out to get the final PCR product encoding C-terminal half of YRS. The randomized mutations were confirmed by sequencing the final PCR products. The mutant library was generated by replacing the C-terminal half of *Sc* YRS gene with the final PCR products by using restriction sites *Bst*XI and *Pst*I (Fig. 1A). The experimental library titer was calculated as  $5.5 \times 10^3$  CFU/ $\mu$ l, which is sufficient enough considering the theoretical maximum diversity of randomizing 6 base pairs, i.e.  $4^6 \cong 4.1 \times 10^3$ .

#### Library screening and characterization of selected clones

Using chloramphenicol resistance derived by amber suppression of CAT amber gene in pACamG, the mutant library was screened on LB plates containing increasing amounts of chloramphenicol. Eight clones were selected from the first screening on LB plates containing 300  $\mu$ g/ml of chloramphenicol. After additional analysis for their chloramphenicol-resistant growth, two positive clones (mYRS-3 and mYRS-5) were finally chosen for further characterization of their amber suppression activity. Assay for the amber suppression-mediated chloramphenicol resistance in the minimal medium of GMML showed two mutant clones had strong resistance at the higher concentrations of

chloramphenicol than wt-YRS (Fig. 2A), and their IC<sub>50</sub> values of chloramphenicol were 122, 286, and 207  $\mu$ g/ml for wt-YRS, mYRS-3, and mYRS-5, respectively (Table 1). For additional *in vivo* amber suppression assay, we have used a reporter strain *E. coli* DH10B (Tn:*lacZam*) which contains a single copy of amber-mutated *lacZ* gene in the genomic DNA (Kim *et al.*, 2009). After co-transforming pACamG and YRS expression plasmids to the strain,  $\beta$ -galactosidase activity was measured from the cell extracts. Compared to wt-YRS, mYRS-3, and mYRS-5 showed approximately 6.5-fold and 2.5-fold higher activities, respectively (Fig. 2B).

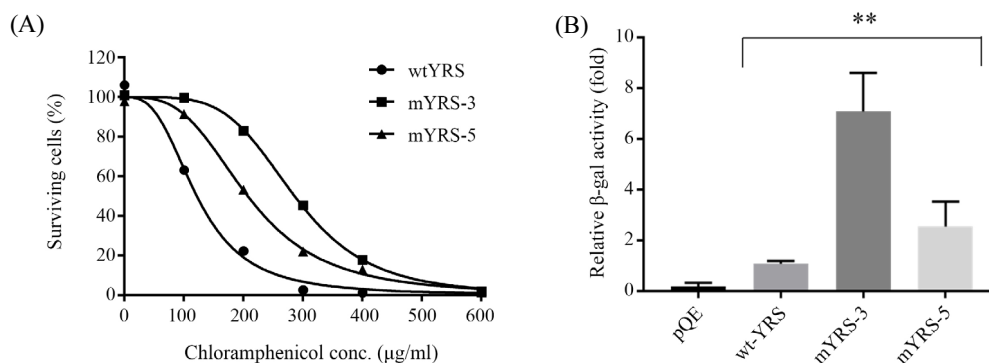
DNA sequencing of the mutant YRSs revealed Pro320 residue has been changed to alanine in both mutants and Asp321 residue to alanine and histidine in mYRS-3 and mYRS-5, respectively (Table 1). P320 residue was known to involve hydrophobic interaction with the base of G34 of tRNA<sup>Tyr</sup> and also participate in a hydrophobic patch composed of Phe254,

**Table 1. Amino acid sequences of mutant YRS and their IC<sub>50</sub> values of chloramphenicol**

	Residue number <sup>a</sup>		Cm IC <sub>50</sub> <sup>b</sup> ( $\mu$ g/ml)
	320	321	
wt YRS	Pro	Asp	122
mYRS-3	Ala	Ala	286
mYRS-5	Ala	His	207

<sup>a</sup> Number of amino acid residues of *Sc* TyrRS

<sup>b</sup> Chloramphenicol concentration showing 50% of growth inhibition in GMML medium



**Fig. 2. Amber suppression activity of mutant YRS clones.** (A) Chloramphenicol-resistant growth derived by amber suppression of CATam gene. Bacterial clones containing pACamG plus either wild type YRS or mutant YRSs selected from library screening were cultured in GMML medium containing increasing amounts of chloramphenicol up to 600  $\mu$ g/ml. After 36 h culture, optical density of the bacterial culture was measured at 600 nm. Relative growth rate was presented as percentile compared to the bacterial growth without chloramphenicol. (B) Comparison of *in vivo*  $\beta$ -galactosidase activity produced by YRS-mediated amber suppression in DH10B (Tn:*lacZam*) strain. The plasmids expressing the indicated YRS were co-transformed with pACamG into *E. coli* DH10B (Tn:*lacZam*) and their amber suppression activities were analyzed by  $\beta$ -galactosidase assay. \*\* $P < 0.01$ .

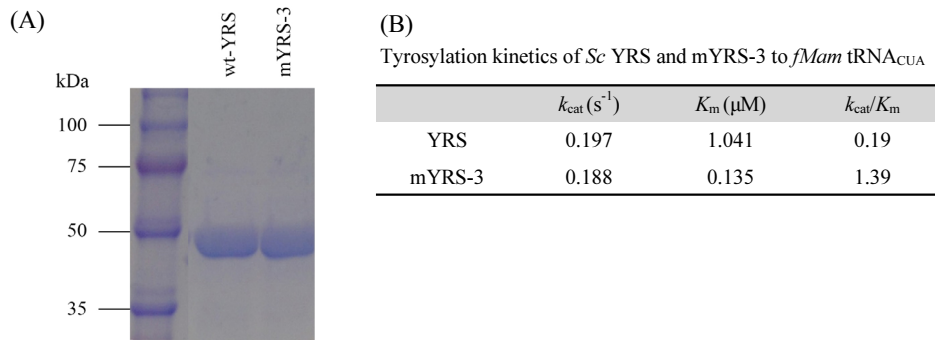
Pro257, Pro319, and Pro320 where accommodates the second and third anticodon bases U35 and A36 in the crystal structure of the ternary complex of *Sc* YRS associated with Tyr-AMP analog and tRNA<sup>Tyr</sup> (Tsunoda *et al.*, 2007). Ala substitution at Pro320 residue may maintain the hydrophobic interaction with the base of C34 of *fMam* tRNA<sub>CUA</sub> and, in addition, confer more flexible and spacious structure for the placement of the other anticodon bases than Pro. On the other hand, Asp321 was known to interact specifically with N1 and O6 atoms of G34 through bifurcated hydrogen bonds, thereby playing as a key residue for specific recognition of G34 (Tsunoda *et al.*, 2007). Similarly, crystal structure of *Mj* YRS complexed with its cognate tRNA and L-tyrosine has shown that Asp286, the residue homologous to Asp321 of *Sc* YRS, interacts with N1 and N2 atoms of G34 by two hydrogen bonds (Kobayashi *et al.*, 2003). In addition, when Asp286 of *Mj* YRS was replaced with other larger amino acids such as glutamine, tyrosine, and arginine residues, the aminoacylation activity of *Mj* YRS mutants for the amber suppressor tRNA which has C34 in place of G34 significantly increased. D286R mutation, which showed the highest activity, exhibited an 8-fold higher activity than the wild type *Mj* YRS (Kobayashi *et al.*, 2003). Those results were well-matched with the fact that cytosine base at position 34 of the amber suppressor tRNA is smaller than guanine. However, inconsistent with *Mj* YRS, our *Sc* YRS mutants showed substitution of Asp321 with alanine or histidine which are not larger amino acids than aspartate. Moreover, D321A mutant which has smaller side chain than histidine exhibited higher *in vivo* amber suppression activity than D321H mutant (Fig. 2). Although the overall anticodon recognition mode of archaeal *Mj* YRS and eukaryotic *Sc* YRS is quite similar, there is a difference in conformation surrounding G34. The hydrophobic interaction between G34 and Pro320 in *Sc* YRS is replaced by the stacking interaction between G34 and His283 in *Mj* YRS. In addition, directed evolution of several UAA-incorporating aaRSs derived from *Mj* YRS for improving their binding affinity to the amber suppressor tRNA revealed that substitutions of amino acids interacting with G34 are different among aaRSs, indicating that the conformation of anticodon recognition pocket may be affected by the structure of the other part of aaRS (Wang *et al.*, 2015). Thus, D321A or D321H mutations in the *Sc* YRS implicate delicate structural differences in the binding pocket of

anticodon as well as the other part of the protein.

Previously, we had generated *Sc* YRS(D321R) mutant by site-directed mutagenesis and tested *in vivo* amber suppression activity of the mutant. In contrast to our expectation, cells transformed with plasmid expressing D321R mutant showed very low level of CAT amber suppression activity (Supplementary data Fig. S1). The low level of amber suppression activity appears to be involved in low copy numbers of D321R mutant expression plasmid because the activity of  $\beta$ -lactamase (the antibiotic resistant marker of D321R mutant expression plasmid) of the same cell extract was also very low. In addition, the expression level of *Sc* YRS(D321R) was also at least 10-fold less than that of wild-type YRS when judged from SDS-PAGE analysis in the purification process of recombinant wt-YRS and YRS(D321R) proteins from the same amount of cell culture (Supplementary data Fig. S2). Taken together, these results suggest that *Sc* YRS(D321R) protein might be toxic to the cells or very labile to be degraded inside cells. Moreover, when we analyzed tyrosylation kinetics of YRS and its D321R mutant to *fMam* tRNA<sub>CUA</sub> with the purified recombinant proteins,  $K_m$  value of D321R mutant was decreased by approximately 2-fold, indicating that D321R mutation increases binding affinity of YRS to *fMam* tRNA<sub>CUA</sub>. D321R mutation increases  $k_{cat}/K_m$  by approximately 2-fold through lowering the  $K_m$  value (Supplementary data Fig. S3). The corresponding D286R mutation of *Mj* YRS increased tyrosylation activity to *Mj* tRNA<sup>Tyr</sup>(G34C) by approximately 65-fold of  $k_{cat}/K_m$  by lowering its  $K_m$  value (Kobayashi *et al.*, 2003). Thus, these results demonstrate D321R mutation of *Sc* YRS is not essential as much as D286R mutation of *Mj* YRS in recognition of C34 in the amber suppressor tRNA, and also support the idea that the delicate structure of anticodon binding pocket of *Sc* YRS may be different from that of *Mj* YRS.

#### Tyrosylation kinetics of wt-YRS and mYRS-3 to *fMam* tRNA<sub>CUA</sub>

To compare tyrosylation activity of wt-YRS and the mutant, wt-YRS and mYRS-3 proteins having an N-terminal 6xHis tag were expressed in *E. coli* M15 strain and purified by immobilized metal affinity chromatography. SDS-PAGE analysis of the purified recombinant proteins showed high quality of purification without any significant contaminant, and production



**Fig. 3. Tyrosylation kinetics of wild type YRS and mYRS-3 to *fMam* tRNA<sub>CUA</sub>.** (A) SDS-PAGE analysis of purified recombinant wt-YRS and mYRS-3 proteins from the same culture volume. (B) Tyrosylation kinetics of wt-YRS and mYRS-3 to the amber suppressor tRNA, *fMam* tRNA<sub>CUA</sub>, were determined from the Lineweaver-Burk reciprocal plot.

yield was almost same between wt-YRS and mYRS-3 (Fig. 3A).

Using the purified wt-YRS and mYRS-3 proteins, we measured their tyrosylation activities with increasing amounts of *fMam* tRNA<sub>CUA</sub>, and their kinetic parameters were determined (Fig. 3B). The mutant enzyme recognized *fMam* tRNA<sub>CUA</sub> approximately 7-fold better than the wt-YRS, which is quite similar to the increase of *in vivo* amber suppression activity measured by  $\beta$ -galactosidase (Fig. 2B). The enhanced aminoacylation activity of the mutant enzyme was mainly due to decrease of the  $K_m$  value for the amber suppressor tRNA because the  $K_{cat}$  values was not significantly different between the wild-type and the mutant.

In conclusion, by using a directed evolution approach, we have generated *Sc* YRS variants which are able to recognize the first anticodon C34 of *fMam* tRNA<sub>CUA</sub> with better affinity. This variant was able to incorporate unnatural amino acids into the protein with significantly enhanced efficiency in response to amber nonsense codon. Thus, the evolved *Sc* YRS mutants can be adopted to general optimization process of unnatural amino acid incorporation in response to amber codon.

## 적 요

효모균 타이로실-tRNA 합성효소(*Sc* YRS)와 엠버 멈춤코돈을 인식하는 대장균 시작tRNA 변이체(*fMam* tRNA<sub>CUA</sub>)쌍은 대장균에서 단백질 생합성시 원하는 특정 위치에 비천연 아미노산을 도입하는데 활용된다. *Sc* YRS/*fMam* tRNA<sub>CUA</sub>쌍의 엠버씨프레션 활성을 높이기 위해 *fMam* tRNA<sub>CUA</sub>의 첫번째 안티코돈 염기를 인식하는 *Sc* YRS의 320번, 321번 아미노

산 잔기를 암호화하는 염기서열을 무작위로 돌연변이시킨 라이브러리를 제작하였다. 엠버씨프레션에 의한 클로람페니콜 저항성을 이용해 라이브러리를 탐색하여 활성이 향상된 2개의 돌연변이주를 선별하였다. 이들의 클로람페니콜 저항성 성장의 IC<sub>50</sub>값은 야생형 YRS보다 1.7~2.3배 높았으며, *in vivo* 엠버씨프레션 활성을 비교한 결과 3~6.5배의 활성 증가가 나타났다. 높은 활성을 보인 mYRS-3 (P320A/D321A) 단백질의 *fMam* tRNA<sub>CUA</sub>에 대한 *in vitro* aminoacylation kinetics 분석은 야생형보다 약 7배 높은 효소활성을 보였으며, 이는 주로 기질인 *fMam* tRNA<sub>CUA</sub>에 대한 결합 친화도가 증가하여 나타났다. 이런 접근법을 이용하여 다양한 종류의 비천연 아미노산 도입에 활용되는 aminoacyl-tRNA 합성효소의 엠버씨프레션 활성을 높임으로써 엠버 멈춤코돈을 이용한 비천연 아미노산 도입 효율성을 높일 수 있을 것이다.

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