Nonsense and Sense Suppression Abilities of Original and Derivative Methanosarcina mazei Pyrrolysyl-tRNA Synthetase-tRNA<sup>Pyl</sup> Pairs in the Escherichia coli BL21(DE3) Cell Strain

Keturah A. Odoi, Ying Huang, Yohannes H. Rezenom, Wenshe R. Liu*

Department of Chemistry, Texas A&M University, College Station, Texas, United States of America

Abstract

Systematic studies of nonsense and sense suppression of the original and three derivative Methanosarcina mazei PyrRStRNA<sup>Pyl</sup> pairs and cross recognition between nonsense codons and various tRNA<sup>Pyl</sup> anticodons in the Escherichia coli BL21(DE3) cell strain are reported. tRNA<sup>Pyl</sup>CUA is orthogonal in E. coli and able to induce strong amber suppression when it is co-expressed with pyrrolysyl-tRNA synthetase (PylRS) and charged with a PyrRS substrate, N<sup>e</sup>-tert-butoxycarbonyl-L-lysine (BocK). Similar to tRNA<sup>Pyl</sup>CUA, tRNA<sup>Pyl</sup>CCU is also orthogonal in E. coli and can be coupled with PyrRS to genetically incorporate BocK at an ochre mutation site. Although tRNA<sup>Pyl</sup>UUA is expected to recognize a UAG codon based on the wobble hypothesis, the PylRS-tRNA<sup>Pyl</sup>UUA pair does not give rise to amber suppression that surpasses the basal amber suppression level in E. coli. E. coli itself displays a relatively high opal suppression level and tryptophan (Trp) is incorporated at an opal mutation site. Although the PylRS-tRNA<sup>Pyl</sup>CCU pair can be used to encode BocK at an opal codon, the pair fails to suppress the incorporation of Trp at the same site. tRNA<sup>Pyl</sup>CCU fails to deliver BocK at an AGG codon when co-expressed with PyrRS in E. coli.

Introduction

Pyrrolysine (Pyl, Figure 1), the 22nd proteinogenic amino acid that was originally discovered in methanogenic methylamine methyltransferase, is genetically encoded by the RNA nucleotide triplet UAG, a stop codon that halts translation of mRNA during a regular protein translation process [1]. The delivery of Pyl to ribosome is mediated by a unique tRNA, tRNA<sup>Pyl</sup>CUA, that is specifically aminoacylated by a unique aminocyl-tRNA synthetase (aaRS), pyrrolysyl-tRNA synthetase (PylRS) [2]. tRNA<sup>Pyl</sup>CUA contains a special nucleotide triplet CUA, an anticodon that recognizes UAG stop codon in mRNA. Unlike tRNA<sup>Sec</sup> that needs a special elongation factor (SecB in E. coli and EFsec in mammalian cells) and an mRNA secondary structure for its binding to the ribosome A site and recognition of a UGA stop codon for the delivery of selenocysteine, tRNA<sup>Pyl</sup>CUA hijacks the regular translation elongation process to suppress a UAG codon for the incorporation of Pyl [3–5]. Previous studies also demonstrated that PyrRS shows remarkably high substrate promiscuity and is able to charge tRNA<sup>Pyl</sup>CUA with a variety of noncanonical amino acids (NAAs). For these reasons and due to the naturally high orthogonality of the PyrRStRNA<sup>Pyl</sup>CUA pair in bacteria, yeast, and mammalian cells, this pair has been directly transferred to E. coli, Saccharomyces cerevisiae, and human cells for the genetic incorporation of more than ten lysine derivatives including N<sup>e</sup>-tert-butoxycarbonyl-L-lysine (BocK) into proteins at amber mutation sites [6–12]. Engineered PyrRS-tRNA<sup>Pyl</sup>CUA pairs have also been used to genetically encode other lysine derivatives and even phenylalanine derivatives that are structurally distinctive from Pyl [13–21]. The genetic incorporation of these NAAs into proteins and their following modifications allow a variety of biochemistry studies such as the functional investigation of protein posttranslational modifications, protein folding dynamic analysis, biosensor development, tracking signal transduction processes, and probing enzyme mechanisms [22].

Citation: Odoi KA, Huang Y, Rezenom YH, Liu WR (2013) Nonsense and Sense Suppression Abilities of Original and Derivative Methanosarcina mazei Pyrrolysyl-tRNA Synthetase-tRNA<sup>Pyl</sup> Pairs in the Escherichia coli BL21(DE3) Cell Strain. PLoS ONE 8(3): e57035. doi:10.1371/journal.pone.0057035

Editor: Lennart Randau, Max-Planck-Institute for Terrestrial Microbiology, Germany

Received November 15, 2012; Accepted January 16, 2013; Published March 8, 2013

Copyright: © 2013 Odoi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Institute of Health (grant 1R01CA161158) and the Welch Foundation (grant A-1715). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: wliu@chem.tamu.edu

PLOS ONE | www.plosone.org 1 March 2013 | Volume 8 | Issue 3 | e57035
was achieved by genetically encoding an evolved MjTyrRS-
$tRNA^{Pyl}_{CUA}$ pair for amber suppression and a wild type or evolved
PylRS-$tRNA^{Pyl}_{CUA}$ pair for ochre suppression in *E. coli*. The genetic incorporation of two different NAAs into one protein can be potentially applied to install a FRET pair in a protein for conformation and dynamic studies as we demonstrated in a separate publication [26], synthesize proteins with two different posttranslational modifications for their functional investigation, and build phage-displayed peptide libraries with the expanded chemical diversities.

Although the PylRS-$tRNA^{Pyl}_{CUA}$ pair has been used extensively for the genetic incorporation of different NAAs in the past few years, two questions related to the pair have not been fully addressed. While our lab and other groups have shown that mutating the anticodon of $tRNA^{Pyl}_{CUA}$ does not significantly affect its interaction with the catalytic domain of PylRS [27], further investigation need to be done to determine whether we can directly use mutant RNA$^{Pyl}$ forms for the genetic incorporation of different NAAs at an opal or ochre codon or even a sense codon in *E. coli*. Another study is necessary to clarify whether an aminoacylated tRNA$^{Pyl}$ can lead to amber suppression since a UUA anticodon can recognize a UAG codon based on the wobble hypothesis [28]. In this study, we attempted to address these two questions and carried out all the experiments in the *E. coli* BL21(DE3) cell strain which has been broadly used for the genetic incorporation of NAAs.

**Materials and Methods**

**Materials**

Phusion high-fidelity DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, and restriction enzymes were purchased from New England Biolabs. Oligonucleotide primers were ordered from Integrated DNA Technologies. Ni-NTA superflow resins were purchased from Qiagen. All polymerase chain reactions (PCRs) were performed using Phusion high-fidelity DNA polymerase. BocK was purchased from Chem Impex. β-Azido-L-phenylalanine (AzF) was synthesized according to a revised literature procedure [29].

**Plasmids**

Plasmid pETrio-pylT(UUA)-PylRS-MCS was derived from pPyRSpylT-GFP1TAG149TAA [25] and carries a $tRNA^{Pyl}_{CUA}$ gene (a C34U mutant form of $tRNA^{Pyl}_{CUA}$) under control of the lpp promoter and the rnc terminator, the wild type *Methanosarcina mazei* PylRS gene under control of the glnS promoter and terminator, and multiple cloning sites including NotI, NdeI, SalI and KpnI targeted sites under control of the T7 promoter and terminator. Plasmid pETrio-pylT(UUA)-PylRS-sfGFP134TAG that carries an additional superfolder green fluorescent protein (sfGFP) gene with an amber mutation at N134 (sfGFP134TAG) was constructed by cloning the sfGFP134TAG gene to the *NsiI* and *KpnI* sites of pETrio-pylT(UUA)-PylRS-MCS.

Three plasmids pETrio-pylT(CUA)-PylRS-sfGFP134TAG, pETrio-pylT(UUA)-PylRS-sfGFP134TAA, and pETrio-pylT(UCA)-PylRS-sfGFP134TGA that vary at the anticodon of $tRNA^{Pyl}$ and have different nonsense mutations at N134 of the sfGFP gene were derived from pETrio-pylT(UUA)-PylRS-sfGFP134TAG. Constructions of these plasmids were carried out using a site-directed mutagenesis protocol that was based on Phusion DNA polymerase. In brief, two oligonucleotide primers, one of which covers the mutation site were used to amplify the whole plasmid of pETrio-pylT(UUA)-PylRS-sfGFP134TAG to give a blunt-end PCR product. This PCR product was phosphorylated by T4 polynucleotide kinase and then self-ligated using T4 DNA ligase. Plasmid pETrio-pylT(CUA)-PylRS-sfGFP134TAG carries genes coding $tRNA^{Pyl}_{CUA}$, PylRS, and sfGFP134TAG; plasmid pETrio-pylT(UUA)-PylRS-sfGFP134TAA carries genes coding$tRNA^{Pyl}_{CUA}$, PylRS, and sfGFP with an ochre mutation at N134 (sfGFP134TAA); and plasmid pETrio-pylT(UCA)-PylRS-sfGFP134TGA carries genes coding$tRNA^{Pyl}_{CUA}$, PylRS, and sfGFP with an opal mutation at N134 (sfGFP134TGA). Plasmid pETrio-pylT(UUA)-PylRS-sfGFP134TAA was derived from plasmid pETrio-pylT(UUA)-PylRS-sfGFP134TAG.

Plasmid pETrio-pylT(CUA)-PylRS-sfGFP134TAG was derived from pETrio-pylT(UUA)-PylRS-sfGFP134TAG by digesting it with *Sphi* to remove the $tRNA^{Pyl}_{CUA}$ gene and parts of the PylRS gene and self-ligating the purified digested plasmid backbone. Plasmid pETrio-pylT(UUA)-PylRS-sfGFP2TG carries genes coding$tRNA^{Pyl}_{CUA}$, PylRS, and sfGFP with an opal mutation at S2 (sfGFP2TG). To construct this plasmid, the sfGFP2TG gene was used to replace the sfGFP134TAG gene in pETrio-pylT(UUA)-PylRS-sfGFP134TAA, and pETrio-pylT(UUA)-PylRS-sfGFP134TGA.

**Amber, Opal, and Ochre Suppression**

Plasmids pETrio-pylT(CUA)-PylRS-sfGFP134TAA, pETrio-pylT(UUA)-PylRS-sfGFP134TGA, and pETrio-pylT(UUA)-PylRS-sfGFP134TAA were individually used to transform *E. coli* BL21(DE3) cells. For each plasmid, a single colony was selected and allowed to grow in 5 mL of LB medium with 100 μg/mL ampicillin at 37°C overnight. The overnight culture was inoculated into 200 mL of 2YT medium with 100 μg/mL ampicillin.

---

**Figure 1. The structures of Pyl, BocK and AzF.**

doi:10.1371/journal.pone.0057035.g001
and allowed to grow at 37°C to OD_{600}~1.2. 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 5 mM BocK were then added to the medium to induce expression of sfGFP. Control experiments in which only 1 mM IPTG was added to the medium were also carried out. The induced cells were allowed to grow at 37°C overnight and then collected by centrifugation (4,200 rpm for 20 min). The collected cells were resuspended in 35 mL of lysis buffer (50 mM HEPES, 300 mM NaCl, 250 mM imidazole, pH 6.1) and lysed by sonication in an ice water bath. The lysed cells were clarified by centrifugation (10,000 rpm for 1 h). The supernatant was decanted and let bind to 5 mL of Ni-NTA superflow resins at 4°C for 1 h. The mixture of the supernatant and resins was then loaded to an empty Qiagen Ni-NTA superflow cartridge. The resins were washed with 5 volume times of lysis buffer and sfGFP was then eluted with buffer (50 mM HEPES, 300 mM NaCl, 250 mM imidazole, pH 6.1). To further purify the expressed sfGFP, the protein was equilibrated against buffer A (20 mM Bis-Tris, pH 6.1) and then loaded to a monoS column (GE Health Science). The protein was eluted out by running a gradient from buffer A to 100% of buffer B (20 mM Bis-Tris, pH 6.1) and then loaded to a monoQ column from GE Health Science. The protein was eluted out by running a gradient from buffer A to 100% of buffer B (20 mM Bis-Tris, pH 6.1). The finally purified protein was then concentrated to a desired volume and analyzed by SDS-PAGE.

To analyze the purified protein by electrospray ionization mass spectrometry (ESI-MS) analysis, the buffer of the purified protein was changed to the phosphate buffer saline. Without further indication, protein purification and characterization in the following experiments were the same.

### Suppression of an Opal Mutation at S2 of sfGFP

Plasmid pETtrio-pylT(UCA)-PylRS-sfGFP2TAG was used to transform E. coli BL21(DE3) cells. A single colony was then used to express sfGFP at two induction conditions: (1) 1 mM IPTG and (2) 1 mM IPTG and 5 mM BocK.

### Basal Suppression at an Opal UGA Codon

Plasmid pETtrio-sfGFP134TGA was used to transform E. coli BL21(DE3) cells. A single colony was then selected to do protein expression that was induced by the addition of 1 mM IPTG.

### Mutagenic Analysis of tRNA_{Pyl}^{\text{CuA}}

Plasmids pETtrio-pylT(UCA)-PylRS-sfGFP134TGA, pETtrio-pylT(UCA)G73C-PylRS-sfGFP134TGA, and pETtrio-pylT(UCA)G73A-PylRS-sfGFP134TGA were used individually to transform E. coli BL21(DE3) cells. A single colony for each plasmid was then selected to do protein expression at two induction conditions: (1) 1 mM IPTG and (2) 1 mM IPTG and 5 mM BocK.

### Anticodon-codon Cross Recognition

Plasmids pETtrio-pylT(UCA)-PylRS-sfGFP134TAG, pETtrio-pylT(UCA)-PylRS-sfGFP134TAA, and pETtrio-pylT(UCA)-PylRS-sfGFP134TGA were individually used to transform E. coli BL21(DE3) cells. A single colony for each plasmid was then selected and allowed to grow in 5 mL of LB medium with 100 μg/mL ampicillin at 37°C overnight. This overnight culture was then inoculated into 500 mL of 2YT medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37°C overnight. This overnight culture was inoculated into 200 mL of 2YT medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and allowed to grow to OD_{600}~1.2. Expression of sfGFP was then induced. Four induction conditions were tested, including (1) 1 mM IPTG only, (2) 1 mM IPTG and 1 mM AzF, (3) 1 mM IPTG and 5 mM BocK, and (4) 1 mM IPTG, 1 mM AzF, and 5 mM BocK.

### AGG Codon Suppression

Plasmid pETtrio-pylT(CCU)-PylRS-sfGFP2AGG was used to transform E. coli BL21(DE3) cells. A single colony was then selected and allowed to grow in 5 mL of LB medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37°C overnight. This overnight culture was then inoculated into 500 mL of 2YT medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and allowed to grow to OD_{600}~1.2. Two conditions were used to induce sfGFP expression. One is the addition of 1 mM IPTG and the other is the addition of 1 mM IPTG and 5 mM BocK.

### Results

#### Amber, Opal, and Ochre Suppression Efficiencies of the PylRS-tRNA_{Pyl} Pairs

To demonstrate amber suppression efficiency of the PylRS-tRNA_{Pyl} pair, E. coli BL21(DE3) cells transformed with pETtrio-pylT(UCA)-PylRS-sfGFP134TAG were used to express sfGFP both in the absence and in the presence of 5 mM BocK, a substitute of PylRS. Without BocK in the growth medium, low level of sfGFP expression was observed. On the contrary, the addition of BocK promoted sfGFP overexpression (Figure 2A). The ESI-MS analysis of the purified sfGFP displayed two major mass peaks at 27,810±1 Da and 27,941±1 Da that agree well with the theoretical molecular weights of sfGFP with BocK incorporated at N134 (27,940 Da for the full-length protein; 27809 Da for the full-length protein without the first methionine (M1)) (Figure 2B1). E. coli BL21(DE3) cells transformed with pETtrio-pylT(UUA)-PylRS-sfGFP134TAA showed an undetectable expression level of sfGFP when BocK was absent in the growth medium. The addition of BocK induced sfGFP expression (Figure 2A). The ESI-MS analysis of the purified sfGFP showed two mass peaks (27,940±1 Da and 27,809±1 Da) that agree well with the theoretical molecular weights of sfGFP with BocK incorporated at N134 (Figure 2B2). In comparison to amber suppression, the expression level of sfGFP using ochre suppression is lower. E. coli BL21(DE3) cells transformed with pETtrio-pylT(UCA)-PylRS-sfGFP134TAG exhibited a high expression level of sfGFP both in the absence and in the presence of BocK in the growth medium (Figure 2A). The ESI-MS analysis of purified sfGFP expressed in the absence of BocK showed a mass peak at 27,899±1 Da that clearly matched a Trp residue at N134 of sfGFP (calculated mass: 27,898 Da) (Figure 2B3). The ESI-MS analysis of sfGFP expressed in the presence of BocK displayed a competitive recognition of the third nucleotide of an amber codon.

Plasmid pEVOL-AzFRS was a gift from Dr. Peter Schultz at Scripps Research Institute [30]. It carries one tRNA^{\text{CuA}} gene under control of a proK promoter and a proK terminator, an evolved AzF-specific MfTyrRS (AzFRS) gene under control of a glnS promoter and a glnS terminator, and an AzFRS gene under control of a pBAD promoter. This plasmid together with pETtrio-pylT(UUA)-PylRS-sfGFP134TAG was used to co-transform E. coli BL21(DE3) cells. One single colony was selected and allowed to grow in 5 mL of LB medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37°C overnight. This overnight culture was inoculated into 200 mL of 2YT medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and allowed to grow to OD_{600}~1.2. Expression of sfGFP was then induced. Four induction conditions were tested, including (1) 1 mM IPTG only, (2) 1 mM IPTG and 1 mM AzF, (3) 1 mM IPTG and 5 mM BocK, and (4) 1 mM IPTG, 1 mM AzF, and 5 mM BocK.
very interesting spectrum. Mass peaks for both Trp residue at N134 of sfGFP (27,900 ± 1 Da) and BocK residue at N134 of sfGFP (27,941 ± 1 Da) were observed. The mass peak for the Trp isoform was much more intensive than the BocK isoform (Figure 2B). To test whether the incorporation of Trp at the 134 position is related to the nucleotide contents of mRNA around the UGA codon, plasmid pETtrio-PylT(UCA)-PylRS-sfGFP134TGA was constructed to test the opal suppression. E. coli BL21(DE3) cells transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA displayed high sfGFP expression levels both in the absence and in the presence of BocK that were much higher than in cells transformed with plasmid pETtrio-PylT(UCA)-PylRS-sfGFP134TAG and grown in the absence of BocK, (B3) transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TAG and grown in the absence of BocK, and (B4) transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA and grown in the presence of 5 mM BocK.

doi:10.1371/journal.pone.0057035.g002

Figure 2. Suppression of amber, opal, and ochre mutations at N134 of sfGFP by their corresponding PylRS-tRNAPyl pairs in the absence and presence of BocK. (A) Proteins shown in the gel represent their real relative sfGFP expression levels. Lanes 1 and 2 were transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TAG; lanes 3 and 4 were transformed with pETtrio-PylT(UUA)-PylRS-sfGFP134TAA; lanes 5 and 6 were transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA. ESI-MS spectra of sfGFP expressed in cells (B1) transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TAG and grown in the presence of 5 mM BocK, (B2) transformed with pETtrio-PylT(UUA)-PylRS-sfGFP134TAA and grown in the presence of 5 mM BocK, (B3) transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA and grown in the absence of BocK, and (B4) transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA and grown in the presence of 5 mM BocK.

doi:10.1371/journal.pone.0057035.g002

A

B

Figure 3. Suppression of amber, opal, and ochre mutations at N134 of sfGFP by their corresponding PylRS-tRNAPyl pairs in the absence and presence of BocK. (A) Proteins shown in the gel represent their real relative sfGFP expression levels. Lanes 1 and 2 were transformed with pETtrio-PylT(CUA)-PylRS-sfGFP134TAG; lanes 3 and 4 were transformed with pETtrio-PylT(UUA)-PylRS-sfGFP134TAA; lanes 5 and 6 were transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA. ESI-MS spectra of sfGFP expressed in cells (B1) transformed with pETtrio-PylT(CUA)-PylRS-sfGFP134TAG and grown in the presence of 5 mM BocK, (B2) transformed with pETtrio-PylT(UUA)-PylRS-sfGFP134TAA and grown in the presence of 5 mM BocK, (B3) transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA and grown in the absence of BocK, and (B4) transformed with pETtrio-PylT(UCA)-PylRS-sfGFP134TGA and grown in the presence of 5 mM BocK.
Figure 3. Suppression of an opal mutation at S2 of sfGFP by the PyrRS-tRNA<sup>Pyf</sup>UCA pair. (A) Expression of sfGFP with an opal mutation. Lanes 1 and 2 were transformed with pETrio-pylT(UCA)-sfGFP134TGA and grown in the absence or presence of 5 mM BocK; lanes 3 and 4 were transformed with pETrio-pylT(UCA)-sfGFP2TGA and grown in the absence or presence of 5 mM BocK. Each protein shown in the gel represents their real relative expression levels. ESI-MS of sfGFP expressed in cells transformed with pETrio-pylT(UCA)-sfGFP2TGA and grown in the (B1) absence or (B2) presence of 5 mM BocK.

doi:10.1371/journal.pone.0057035.g003

Figure 4. Suppression of an opal mutation at N134 of sfGFP at different conditions. (A) Proteins shown in the gel represent their real relative expression levels. Lane 1 was transformed with pET-sfGFP134TGA; lanes 2 and 3 were transformed with pET-pylT(UCA)-sfGFP134TGA and grown in the absence or presence of 5 mM BocK. (B) ESI-MS of sfGFP expressed in cells transformed with pETrio-sfGFP134TGA.

doi:10.1371/journal.pone.0057035.g004
The suppression efficiencies of three mutated forms of \( tRNA_{\text{UCA}}^{\text{Pyl}} \) with mutations as G73A, G73C, and G73U, respectively were also examined. Cells transformed with either pETtrio-pylT(UCA)G73C-PylRS-sfGFP134TGA or pETtrio-pylT(UCA)G73C-PylRS-sfGFP134TGA displayed similar expression levels of sfGFP both in the presence and in the absence of BocK. However, cells transformed with pETtrio-pylT(UCA)G73U-PylRS-sfGFP134TGA showed significantly different expression levels of sfGFP when grown in the absence and in the presence of BocK. The ESI-MS analysis of sfGFP expressed in the absence of BocK showed a major mass peak at 27,895±1 Da that matches sfGFP with Trp incorporated at N134. The addition of 5 mM BocK promoted the sfGFP expression level to increase. The ESI-MS analysis of the purified protein confirmed sfGFP with BocK incorporated at N134 became dominant. The intensity of the mass peak at 27,940±1 Da that matches sfGFP with BocK incorporated at N134 is roughly twice of the mass peak at 27,898±1 Da that matches sfGFP with Trp incorporated at N134.

Anticodon-codon Cross Recognition

Transforming E. coli BL21(DE3) cells with pETtrio-pylT(CUA)-PylRS-sfGFP134TAA followed by growing cells in the presence of 5 mM BocK did not lead to detectable expression of sfGFP. E. coli BL21(DE3) cells transformed with pETtrio-pylT(UUA)-PylRS-sfGFP134TGA showed a detectable but low level of sfGFP expression (less than 1 mg/L) (Figure 6). To see whether BocK promoted suppression at the amber mutation site, E. coli BL21(DE3) cells transformed with pETtrio-pylT(UUA)-PylRS-sfGFP134TGA was also grown in the absence of BocK. As shown in Figure 7A, the sfGFP expression levels both in the absence and in the presence of BocK were very similar. Addition of BocK did not lead to significant increase of amber suppression. As shown in Figures 7B1&B2, sfGFP expressed in both conditions displayed mass peaks (27,839±1 Da and 27,710±1 Da in Figure 7B1 and 27,840±1 Da and 27,709±1 Da in Figure 7B2) that match sfGFP with glutamic acid (Glu), lysine (Lys), or glutamine (Gln) incorporated at N134. Figure 7B2 did show a mass peak at 27,939±1 Da that matches the molecular weight of sfGFP with BocK incorporated at N134. However, its intensity was much lower than the mass peak of sfGFP at ~27,840±1 Da.

Experiments were also carried out to demonstrate that the PylRS-\( tRNA_{\text{UCA}}^{\text{Pyl}} \) pair does not interfere with suppression of an amber mutation mediated by an evolved MjTyRS-\( tRNA_{\text{CUA}}^{\text{Tyr}} \) pair. Two plasmids, pETtrio-pylT(UUA)-pylRS-sfGFP134TGA and pEVOL-AzFRS were used to transform E. coli BL21(DE3)-cells. As shown in Figure 8A, growing the transformed cells in four conditions led to different expression levels of sfGFP. When no NAA or only 5 mM BocK was provided in the medium, only a detectable but very low level of sfGFP expression (less than 1 mg/L) was detected. However, addition of 1 mM AzF to the medium supplemented with or without BocK promoted sfGFP overexpression. The ESI-MS analysis of purified sfGFP in all four conditions

**Figure 5.** Suppression of an opal mutation at N134 of sfGFP by different \( tRNA_{\text{UCA}}^{\text{Pyl}} \) variants. (A) Proteins shown in the gel represent their real relative expression levels. Lanes 1 and 2 were transformed with pETtrio-pylT(UCA)G73C-sfGFP134TGA and grown in the absence or presence of 5 mM BocK; lanes 3 and 4 were transformed with pETtrio-pylT(UCA)G73A-sfGFP134TGA and grown in the absence or presence of 5 mM BocK; lanes 5 and 6 were transformed with pETtrio-pylT(UCA)G73U-sfGFP134TGA and grown in the absence or presence of 5 mM BocK; lanes 7 and 8 were transformed with pETtrio-pylT(UCA)-sfGFP134TGA and grown in the absence or presence of 5 mM BocK. The ESI-MS analysis of sfGFP expressed in cells transformed with pETtrio-pylT(UCA)G73U-sfGFP134TGA and grown in the (B1) absence or (B2) presence of 5 mM BocK. doi:10.1371/journal.pone.0057035.g005
displayed very interesting spectra (Figure 8B). Although a mass peak for sfGFP expressed in two conditions with the supplement of AzF (27,900 ± 1 Da in Figure 8B2 and 27,901 ± 1 Da in Figure 8B4) matches the calculated molecular weight (27,901 Da) of sfGFP with AzF incorporated at N134, a mass peak for sfGFP expressed in two conditions without the supplement of AzF (27,859 ± 1 Da in Figure 8B1 and 27,860 ± 1 Da in Figure 8B3) does not match the molecular weight of sfGFP with either Lys, Glu, or Gln residue at the amber mutation site. Since PyrS does not recognize Lys, Glu, and Gln and tRNAPylCUA itself does not mediate detectable amber suppression in the E. coli Top10 cell strain (data not shown), this low but detectable sfGFP expression level was due to the basal amber suppression in the E. coli BL21(DE3) cell strain [6,27]. This basal amber suppression that was also demonstrated in one separate study from us [27] arises possibly from the recognitions of the UAG codon by its near-cognate tRNAs including tRNAlysUUU/tRNAgluUUC/tRNAGlnCUG [31,32].

A similar test with E. coli BL21(DE3) cells transformed with pETtrio-pyTrT(UCA)-PylRS-sfGFP134TGA and grown in the absence of BocK yielded a sfGFP expression level of 8 mg/L. The ESI-MS spectrum of the purified protein showed a Trp residue at N134 of sfGFP. To rule out the possibility that tRNApylUCA was charged by E. coli tryptophanyl-tRNA synthetase.

Figure 6. Cross recognitions between different anticodons of tRNAPyl and nonsense mutations at N134 of sfGFP. Cells were transformed with pETtrio-PylT(NNN)-PylRS-sfGFP134N’N’N’ and grown in the presence of 5 mM BocK (NNN and N’N’ denote anticodons and codons specified in the figure). Proteins shown in the gel represent their real relative expression levels.
doi:10.1371/journal.pone.0057035.g006

AGG Codon Suppression

E. coli BL21(DE3) cells transformed with pETtrio-pyTrT(CCU)-PylRS-sfGFP2AGG showed similar sfGFP expression levels both in the absence and in the presence of BocK (Figure 9A). sfGFP proteins expressed in both conditions displayed one major mass peak at 27,895 Da that agrees well with the theoretical molecular weight of sfGFP with arginine (Arg) incorporated at N134 (calculated mass: 27,896 Da for the full-length protein) (Figure 9B).

Discussion

Bacterial Nonsense Suppression in the E. coli BL21(DE3) Cell Strain

E. coli BL21(DE3) cells transformed with pETtrio-pyTrT(CUA)-PylRS-sfGFP134TAG and grown in the absence of BocK yielded a sfGFP expression level close to 1 mg/L. The ESI-MS spectrum of the purified sfGFP clearly indicated a Lys, Glu, or Gln residue at the amber mutation site. Since PyrS does not recognize Lys, Glu, and Gln and tRNAPylCUA itself does not mediate detectable amber suppression in the E. coli Top10 cell strain (data not shown), this low but detectable sfGFP expression level was due to the basal amber suppression in the E. coli BL21(DE3) cell strain [6,27]. This basal amber suppression that was also demonstrated in one separate study from us [27] arises possibly from the recognitions of the UAG codon by its near-cognate tRNAs including tRNAlysUUU/tRNAGluUUC/tRNAGlnCUG [31,32].

A similar test with E. coli BL21(DE3) cells transformed with pETtrio-pyTrT(UUA)-PylRS-sfGFP134TAG and grown in the absence of BocK yielded a sfGFP expression level of 8 mg/L. The ESI-MS spectrum of the purified protein showed a Trp residue at N134 of sfGFP. To rule out the possibility that tRNApylUCA was charged by E. coli tryptophanyl-tRNA synthetase.
(TrpRS) for the delivery of Trp at the designated opal mutation site, we constructed plasmid pETtrio-sgFP134TGA that did not contain the tRNA\textsubscript{Pyl\textsubscript{UCA}} gene for further tests. E. coli BL21(DE3) cells transformed with this plasmid yielded a sfGFP expression level of 4 mg/L. The ESI-MS spectrum of the purified protein also showed the incorporation of Trp at N134 of sfGFP. Since we recently demonstrated that tRNA\textsubscript{Pyl\textsubscript{UCA}} is not recognized by E. coli TrpRS, [27] we think sfGFP expressed in the above two conditions resulted from the basal opal suppression in the E. coli BL21(DE3) cell strain. The observed different sfGFP expression levels might be caused by different copy numbers of two plasmids in the transformed cells. This basal opal suppression is the consequence of the recognition of the opal codon by tRNA\textsuperscript{Trp}. E. coli tRNA\textsuperscript{Trp} has a CCA anticodon which is a near-cognate tRNA of UGA. It forms two Watson-Crick base pairs with the 5' and middle nucleotides of UGA and a wobble base pair with the 3' nucleotide of UGA, explaining its ability to recognize UGA. We have also excluded the possibility that nucleotide contents around the opal mutation at N134 of sfGFP facilitated the binding of tRNA\textsuperscript{Trp} and induced the Trp incorporation at this site. The observed sfGFP expression level in E. coli BL21(DE3) cells reached to 115 mg/L. This is roughly 20% of the expression level of wild-type sfGFP in E. coli BL21(DE3) cells (~500 mg/L, unpublished data). This high efficiency to read across an opal codon with the binding of a near-cognate tRNA\textsuperscript{Trp} may correlate with the short distance from the opal codon to the start codon.

In contrary to UAG and UGA codons, the UAA codon displays high translation termination stringency in the E. coli BL21(DE3) cell strain. Cells transformed with pETtrio-pylT(UCA)-PylRS-sgFP2TGA showed an undetectable basal ochre suppression level. This can be explained from several aspects. UAG and UGA are recognized by release factor 1 and release factor 2, respectively, whereas UAA is recognized by both release factor 1 and release factor 2. Its recognition by both release factor proteins, in theory, makes the translation termination at UAA more stringent than the other two stop codons. Another reason lies at the nucleotide contents of UAA. Unlike UAG and UGA that

![Figure 8. Expression of sfGFP in cells transformed with pETtrio-PylT(UUA)-PylRS-sgFP134TAG and pEVOl-AzFRS. (A) Cells were grown in 2YT medium supplemented with different combinations of NAAs. ESI-MS of sfGFP expressed in the (B1) absence of both AzF and BocK; (B2) presence of 1 mM AzF; (B3) presence of 5 mM BocK; and (B4) presence of both 1 mM AzF and 5 mM BocK.

![Figure 9. Suppression of an AGG mutation at S2 of sfGFP by tRNA\textsubscript{Pyl\textsubscript{CCU}}. (A) Expression of sfGFP in cells transformed with pETtrio-pylT(CC\textsubscript{UC})-sgFP2AGG and grown in the absence or presence of 5 mM BocK. (B) The ESI-MS analysis of sfGFP expressed in the presence of 5 mM BocK.

"PLOS ONE | www.plosone.org 8 March 2013 | Volume 8 | Issue 3 | e57035"
could involve a GC base pair interaction, UAA could only form AU pairs or wobble pairs. Its interactions with tRNAs are relatively weak, making its misrecognition less possible than UAG and UGA.

Amber, Opal, and Ochre Suppression Efficiencies of the PylRS-tRNA\(^{Pyl}\) Pairs

When co-expressed with PylRS, all three tRNA\(^{Pyl}\) isoforms \(tRNA_{73}^{Pyl}(UUA)\), \(tRNA_{73}^{Pyl}(UUA)\), and \(tRNA_{73}^{Pyl}(UUA)\) are capable to deliver BocK at their corresponding codon sites. \(tRNA_{73}^{Pyl}(UUA)\) is orthogonal in \(E. coli\) and displays the highest efficiency in all three isoforms. Given that \(E. coli\) BL21(DE3) cells transformed with pET trio-pylT(UUA)-PylRS-sfGFP134TAA did not show a detectable expression level of sfGFP in the absence of BocK, we could conclude that \(tRNA_{73}^{Pyl}(UUA)\) is fully orthogonal in \(E. coli\). In comparison to sfGFP expressed in cells transformed with pET trio-pylT(CUA)-PylRS-sfGFP134TAG and grown in the presence of BocK, the sfGFP expression level in cells transformed with pET trio-pylT(UUA)-PylRS-sfGFP134TAG and grown in the presence of BocK is five times lower. The low ability of \(tRNA_{73}^{Pyl}\) to deliver BocK is possibly due to the relative weak base pair interactions between its UUA anticodon, its UAA stop codon and the availability of both release factor 1 and release factor 2 to stop the translation at a UAA stop codon. In any case, the ochre suppression level achieved by the PylRS-tRNA\(^{Pyl}\) pair is sufficient to promote overexpression of a protein with an ochre mutation. Although a BocK-aminoacylated \(tRNA_{73}^{Pyl}(UUA)\) is able to suppress an opal codon for the incorporation of BocK, it does not inhibit the high incorporation of Trp at the same site. As an exogenous tRNA, the sequence and structure of \(tRNA_{73}^{Pyl}(UUA)\) may not be optimal for the protein translation process in \(E. coli\). When facing a competition from \(E. coli\) tRNA\(^{Trp}\), the recognition of \(tRNA_{73}^{Pyl}(UUA)\) by \(E. coli\) translation machinery may be inhibited. Since the 73rd nucleotide serves as a strong recognition element for most tRNAs [33], it was mutated in \(tRNA_{73}^{Pyl}(UUA)\) to U, A, and G and searched for a mutant that shows a higher ochre suppression efficiency. When co-expressed with PylRS in the presence of BocK, \(tRNA_{73}^{Pyl}(G73U)\) led to higher incorporation level of BocK compared to Trp at the same opal mutation site. However, further mutagenesis with \(tRNA_{73}^{Pyl}(G73U)\) is necessary to fully inhibit the incorporation of Trp at an opal mutation site.

Anticodon-codon Cross Recognition

The wobble hypothesis was first introduced by Francis Crick in 1966 to explain the observation that a single tRNA is able to efficiently recognize multiple codons [28]. Based on this hypothesis, an ochre suppressor tRNA\(^{UUA}\) is also capable of recognizing an amber UAG codon. This is a concern when both UAG and UAA codons are used to code two different NAs. However, cells transformed with pET trio-pylT(UUA)-PylRS-sfGFP134TAG and grown in the presence of 3 mM BocK showed a sfGFP expression level close to that from the basal amber suppression. This suggests very weak recognition of UAG by tRNA\(^{UUA}\). Weak base pairing interactions involved with the UUA anticodon may contribute to the weak recognition of UAG. However, this is certainly not the determining factor since other tRNAs such as tRNA\(^{UUA}\) is also involved in wobble base pairing interactions to recognize multiple codons. One possible explanation for this weak recognition of UAG by tRNA\(^{UUA}\) is the tRNA modifications. All cognate tRNAs are known to exhibit similar affinities for the ribosome A site when they bind to corresponding codons [34,35]. This uniform binding is unexpected as certain codon-anticodon interactions are expected to be more stable than others due to factors such as the GC base pair content. It has been proposed that the specific sequence and post-transcriptional modification status of the tRNA in the region near the anticodon is tuned to ensure nearly indistinguishable binding of tRNAs to the ribosome A site [36–38]. This has been the case for tRNAs such as tRNA\(^{UUA}_{73}\) in which both nucleotides at 34 and 37 are post-transcriptionally modified to achieve similar recognition of AAA and AAG codons [38]. Unlike endogenous tRNAs that have corresponding modification enzymes, tRNA\(^{UUA}_{73}\) is exogenous and may not be targeted by tRNA modification enzymes in \(E. coli\). \(tRNA_{73}^{Pyl}\) without modifications at its anticodon loop likely has a very weak binding affinity to the ribosome A site to associate UAG. Our finding points out that wobble base pairing at the 3' nucleotide of a codon is not sufficient for recruiting a tRNA to the ribosome A site. Additional interactions are required. This aspect needs to be further investigated. Since tRNA\(^{Pyl}\) has a low ability to recognize UAG, it is feasible to use an amber suppressor aaRS-tRNA pair and a wild type or evolved PylRS-tRNA\(^{Pyl}\) pair to code two different NAs at amber and ochre mutation sites, respectively, in \(E. coli\).

During the anticodon-codon cross recognition analysis to examine whether tRNA\(^{Pyl}\) can compete against tRNA\(^{UUA}\) to bind UAG in the ribosome A site, we noticed that Phe was incorporated at N134 of sfGFP which was expressed in cells transformed with pEVO-LysFRS and pET trio-pylT(UUA)-PylRS-sfGFP134TAG and grown either in the absence or presence of BocK. We think AzF can recognize Phe, leading to the misincorporation of Phe. Since AzF is a hydrophobic amino acid and structurally very similar to Phe, one would expect that AzF-tRNA that was originally evolved from MjTyrRS would probably recognize Phe at a relatively low level and charge tRNA\(^{Pyl}\) with Phe when AzF is absent in the medium. Although not clearly addressed in existing literature, most evolved MjTyrRS-tRNA\(^{UUA}\) pairs did show significant background amber suppression even in minimal media [39,40]. Since most evolved MjTyrRS variants are for Phe derivatives, it is highly possible that background amber suppression caused by these evolved MjTyrRS-tRNA\(^{UUA}\) pairs was due to their recognition of either Phe or Tyr or both. In this study, the background amber suppression induced by the AzF-tRNA\(^{Pyl}\) pair inhibited both the basal amber suppression level and amber suppression induced by the PylRS-tRNA\(^{Pyl}\) pair. This test also provided evidence that amber suppression mediated by the PylRS-tRNA\(^{Pyl}\) pair is too low to be a concern. It also points out that substrate specificities of evolved NAA-specific aaRSs need to be further characterized.

AGG Codon Suppression

Our lab and other groups have shown that mutating the anticodon of tRNA\(^{Pyl}\) does not significantly affect its interactions with PylRS. Three tRNA\(^{Pyl}\) isoforms that are specific for three stop codons are capable to deliver BocK at their corresponding codon sites when co-expressed with PylRS. We were also curious about the ability a tRNA\(^{Pyl}\) isoform to suppress a sensing codon. We chose to test on the suppression of the AGG codon since it is a rarely used codon and tRNA\(^{AGG}_{73}\) is limitedly expressed in the \(E. coli\) BL21(DE3) cell strain [41]. However, \(E. coli\) transformed with pET trio-pylT(CCU)-PylRS-sfGFP2AGG only expressed sfGFP with Arg at its S2 position in the presence of 5 mM BocK.
Increasing the BocK concentration to 10 mM did drive the expression of sfGFP with BocK incorporated at S2 of sfGFP, which was confirmed by the ESIMS analysis of the purified proteins (data not shown). However, in comparison to the Arg-containing sfGFP isoform that showed a high intensity in the ESIMS spectrum of the purified sfGFP, the intensity for the BocK-containing sfGFP isoform was very low. This indicates tRNA\textsubscript{Pyl}\textsubscript{CCU} is charged by PylRS with BocK in E. coli and is able to deliver BocK at an AGG codon site. However, the PyrRS-tRNA\textsubscript{Pyl}\textsubscript{CCU} pair cannot compete efficiently against the endogenous Arg incorporation system at the AGG codon. Similar to tRNA\textsubscript{Pyl}\textsubscript{CCU}\textsubscript{UAU}, the sequence and structure of tRNA\textsubscript{Pyl}\textsubscript{CCU}\textsubscript{UAU} may not be optimized for the protein translation machinery in E. coli.

In summary, the suppression efficiencies of the original and three tRNA\textsubscript{Pyl} variant, the cross recognition between nonsense codons and tRNA\textsubscript{Pyl} anticodons in the E. coli BL21(DE3) cell strain have been investigated. Among all tRNA\textsubscript{Pyl} isoforms, tRNA\textsubscript{Pyl}\textsubscript{CCU}\textsubscript{UAU} has the highest suppression efficiency for the delivery of BocK at its corresponding codon. Besides its orthogonal nature in E. coli, tRNA\textsubscript{Pyl}\textsubscript{CCU}\textsubscript{UAU} does not induce a significant level of suppression at an amber codon. This is contrary to the wobble hypothesis and makes it feasible to use amber suppressing aaRS-tRNA pair and the PyrRS-tRNA\textsubscript{Pyl}\textsubscript{UCU} pair to code two different NAAs at amber and ochre codons respectively in E. coli. Our study also demonstrates the PyrRS-tRNA\textsubscript{Pyl}\textsubscript{UCU} pair cannot efficiently deliver BocK at an AGG codon site. Further work to optimize the sequence and structure of tRNA\textsubscript{Pyl}\textsubscript{CCU}\textsubscript{UAU} for the E. coli translation machinery may be necessary to increase the BocK incorporation efficiency and suppress the Arg incorporation at the AGG codon.

Acknowledgments
We thank Dr. Ryan Mehl at Franklin & Marshall College for providing us plasmids pBAD-sfGFP and pBAD-sGFP134TAG and Dr. Peter G. Schultz at the Scripps Research Institute for providing us the plasmid pEVOL-AeFRS.

Author Contributions
Conceived and designed the experiments: KAO YH YR WRL. Performed the experiments: KAO YH YR. Analyzed the data: KAO YH. Contributed reagents/materials/analysis tools: KAO YH. Wrote the paper: WRL.

References
1. Hao B, Gong W, Ferguson TK, James CM, Kryzwicki JA, et al. (2002) A New UAG-Encoded Residue in the Structure of a Methanogen Mutatransferase. Science 296: 1459–1462.
2. Srinivasan G, James CM, Kryzwicki JA (2002) Pyrrolyl-Tyr synthetase encoded by UAG: a cellular mechanism that protects against the toxic effects of a nonsense amino acid. J Cell Biol 158: 1055–1064.
3. Kozak M (1991)Scan for efficient translation initiation sites in mammalian mRNA.
4. Yuan J, O’Donoghue P, Ambrogelli A, Gundellapalli S, Sherrer RL, et al. (2010) Engineering pyrrolysyl-tRNA synthetases. Biochem Biophys Res Commun 371: 159–163.
5. Yuan J, O’Donoghue P, Ambrogelli A, Gundellapalli S, Sherrer RL, et al. (2010) Distinct genetic code expansion strategies for selenocysteine and pyrroline are reflected in different aminoacyl-tRNA formation systems. FEBS Lett 594: 342–349.
6. Poylarco CR, Herrings S, Berhave A, Wood JL, Soll D, et al. (2006) Pyrroline analogues as substrates for pyrrolysyl-tRNA synthetase. FEBS Lett 580: 6695–6699.
7. Mukai T, Kobayashi T, Hino N, Yanagisawa T, Sakamoto K, et al. (2008) Aminoacylation of pyrroline-2-carboxylic acid by engineered tRNA\textsubscript{Pyl} synthases. Biochem Biophys Res Commun 371: 818–822.
8. Yanagisawa T, Ishii R, Fukumaga R, Kobayashi T, Sakamoto K, et al. (2008) Mutistep engineering of pyrrolysyl-tRNA synthetase to genetically encode \textit{N}(\textit{epsilon})-(\textit{o}-azidobenzyloxycarbonyl) lysine for site-specific protein modification. Chem Biol 15: 1187–1197.
9. Nguyen DP, Luic H, Neumann H, Kapudian PB, Deiters A, et al. (2009) Genetic encoding and labeling of aliphatic azides and alkynes in recombinant proteins via a pyrrolysyl-tRNA synthetase/tRNA(UUA) pair and click chemistry. J Am Chem Soc 131: 8720–8721.
10. Ai HW, Lee JW, Schultz PG (2010) A method to site-specifically introduce methyleneides into proteins in E. coli. Chem Commun (Camb) 6: 5306–5308.
11. Feinberg T, Li X, Lee MM, Chao MK (2009) A pyrroline analogue for protein click chemistry. Angew Chem Int Ed 48: 1635–1638.
12. Li X, Feinberg T, Ottesen JJ, Chao MK (2009) A pyrroline analogue for site-specific protein ubiquitination. Angew Chem Int Ed 48: 9184–9187.
13. Neumann H, Peak-Chew SY, Chin JW (2008) Genetically encoding N(\textit{epsilon})-methyl-L-lysine. Mol Biosyst 6: 1557–1560.
14. Huang Y, Wan W, Russell WK, Pai PJ, et al. (2010) A genetically encoded photoreactive N(\textit{epsilon})-(\textit{o}-azidobenzyloxycarbonyl) lysine for site-specific protein modification. Biochem Biol 15: 1187–1197.
15. Nguyen DP, Luic H, Neumann H, Kapudian PB, Deiters A, et al. (2009) Genetic encoding and labeling of aliphatic azides and alkynes in recombinant proteins via a pyrrolysyl-tRNA synthetase/tRNA(UUA) pair and click chemistry. J Am Chem Soc 131: 8720–8721.
16. Ai HW, Lee JW, Schultz PG (2010) A method to site-specifically introduce methyleneides into proteins in E. coli. Chem Commun (Camb) 6: 5306–5308.
17. Zhang M, Liu S, Song X, Liu J, Fu Y, et al. (2011) A genetically incorporated crosslinker reveals chaperone cooperation in acid resistance. Nat Chem Biol 7: 671–677.
18. Takimoto JK, Delis N, Noel JP, Wang L (2011) Stereochemochemical basis for engineered pyrrolysyl-tRNA synthetase and the efficient in vivo incorporation of structurally divergent non-native amino acids. ACS Chem Biol 6: 743–745.
19. Wang YS, Fang X, Wallace AL, Wu B, Liu WR (2012) A rationally designed pyrrolysyl-tRNA synthetase mutant with a broad substrate spectrum. J Am Chem Soc 134: 2950–2953.
20. Gautier A, Nguyen DP, Luic H, An W, Deiters A, et al. (2010) Genetically encoded photocoupler of protein localization in mammalian cells. J Am Chem Soc 132: 4086–4092.
21. Liu WR, Wang YS, Wang W (2011) Synthesis of proteins with defined posttranslational modifications using the genetic noncanonical amino acid incorporation approach. Mol Biosyst 7: 38–47.
22. Anderson JC, Wu N, Santoro SW, Lakshman V, King DS, et al. (2004) An expanded genetic code with a functional quadruplet codon. Proc Natl Acad Sci U S A 101: 7566–7571.
23. Neumann H, Wang Y, Davis L, Garcia-Alain M, Chin JW (2010) Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome. Nature 464: 441–444.
24. Wan W, Huang Y, Wang Z, Russell WK, Pai PJ, et al. (2010) A facile system for genetic incorporation of two different noncanonical amino acids into one protein in Escherichia coli. Angew Chem Int Ed 49: 3211–3214.
25. Wu B, Wang Z, Huang Y, Liu WR (2012) Catalyst-free and site-specific one-pot dual-labeling of a protein directed by two genetically incorporated noncanonical amino acids. Chembiochem 13: 1405–1408.
26. O’Donoghue P, Prat I, Heinemann HU, Liang J, Oldi K, et al. (2012) Near-cognate suppression of amber, opal and quadruplet codons competes with aminoacyl-tRNA/Pyl for genetic code expansion. FEBS Lett 586: 3931–3937.
27. Crick FH (1966) Codon-anticodon pairing: the wobble hypothesis. J Mol Biol 19: 549–553.
28. Schwizer R, Caviezel M (1971) p-Azido-L-phenylalanine: A photo-affinity ‘probe’ related to tyrosine. Helvetica Chimica Acta 54: 1395–1400.
29. Young TS, Ahmad I, Yin JA, Schultz PG (2010) An enhanced system for unnatural amino acid mutagenesis in E. coli. J Mol Biol 395: 361–374.
30. Swanson R, Hohen P, Sumner-Smith M, Uermura H, Watson L, et al. (1988) Accuracy of in vivo aminoacylation requires proper balance of RNA and aminoacyl-tRNA synthetase. Science 242: 1548–1551.
31. Fukumaga R, Ohno S, Nishikawa K, Yokogawa T (2007) A base pair at the bottom of the anticondon stem is reciprocally preferred for discrimination of cognate tRNAs by Escherichia coli 5S- and glutaminyl-tRNA synthetases. Nucleic Acids Res 35: 3108–3110.
32. Kurata HS, Green R (2009) Fidelity at the Molecular Level: Lessons from Protein Synthesis. Cell 136: 746–762.
33. Agris PF, Vendeix FA, Graham WD (2007) tRNAs wobble decoding of the genome: 40 years of modification. J Mol Biol 366: 1–13.
37. Allner O, Nilsson I (2011) Nucleotide modifications and tRNA anticodon-mRNA codon interactions on the ribosome. RNA 17: 2177-2188.
38. Vold BS, Keith DE, Jr., Buck M, McCloskey JA, Pang H (1982) Lysine tRNAs from Bacillus subtilis 168: structural analysis. Nucleic Acids Res 10: 3125-3132.
39. Wang L, Brock A, Herberich B, Schultz PG (2001) Expanding the genetic code of Escherichia coli. Science 292: 498-500.
40. Xie J, Wang L, Wu N, Brock A, Spraggon G, et al. (2004) The site-specific incorporation of p-iodo-L-phenylalanine into proteins for structure determination. Nat Biotechnol 22: 1297-1301.
41. Gribskov M, Devereux J, Burgess RR (1984) The codon preference plot: graphic analysis of protein coding sequences and prediction of gene expression. Nucleic Acids Res 12: 539-549.