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Genetic-code evolution for protein synthesis with non-natural amino acids

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ABSTRACT

The genetic encoding of synthetic or "non-natural" amino acids promises to diversify the functions and structures of proteins. We applied rapid codon-reassignment for creating *Escherichia coli* strains unable to terminate translation at the UAG "stop" triplet, but efficiently decoding it as various tyrosine and lysine derivatives. This complete change in the UAG meaning enabled protein synthesis with these non-natural molecules at multiple defined sites, in addition to the 20 canonical amino acids. UAG was also redefined in the *E. coli* BL21 strain, suitable for the large-scale production of recombinant proteins, and its cell extract served the cell-free synthesis of an epigenetic protein, histone H4, fully acetylated at four specific lysine sites.

1. Introduction

Codon assignment in the genetic code is redundant. Each of the 20 canonical amino acids, except for methionine and tryptophan, and the translation stop have more than one codon, and thus some of the synonymous codons could be reassigned to synthetic or "non-natural" amino acids [1]. Although such amino acids have been encoded genetically [2–4], their assigned codons are still recognized by the endogenous translation factors, and they retain their original identities. This ambiguity in codon assignment has limited the applicability of non-natural amino acids. When stop or quadruplet codons are assigned to the molecules, their incorporation into proteins is hampered by the release factors recognizing the stop codon, or the endogenous tRNA species reading the first three letters of the quadruplet codon [3,4]. Otherwise, the identical sense codons are assigned to non-natural and canonical amino acids [2]; the efficient incorporation of the non-natural amino acid inevitably causes the total replacement of the canonical amino acid in recombinant proteins and the whole proteome.

We previously demonstrated the feasibility of completely reassigning a codon *in vivo*, by eliminating the endogenous factor recognizing it [5]. The amber UAG triplet, one of the three stop codons, was thus reassigned to canonical amino acids or even a non-natural molecule, 3-iodo-L-tyrosine (Fig. 1A), in an *Escherichia coli* strain with the knocked-out *prfA* gene. This gene encodes release factor 1, the essential cellular component recognizing UAG and terminating translation. Although the *E. coli* strain with the UAG reassignment to glutamine grew almost as vigorously as the parent strain K-12 HST08, the UAG reassignment to the non-natural molecule seriously reduced growth rate and poorly supported the large-scale production of proteins with the amino acid. In the present study, we identified a key gene for enhancing the viability of UAG-reassigned *E. coli* or "RFzero" strains. This finding helped us to redefine UAG with various non-natural amino acids in a number of *E. coli* strains with diverse genetic backgrounds, and achieve large-scale protein production with these molecules at defined multiple sites.

2. Materials and methods

2.1. E. coli strains and growth media

HST08 was purchased from Takara Bio (Japan). BL21(DE3) and HMS174(DE3) were from Novagen. BW25113 was provided by the National BioResource Project (Japan). 3-Iodo-L-tyrosine (Sigma–Aldrich) and 4-azido-L-phenylalanine (Bachem) were dissolved in 0.2 N HCl and added to Luria–Bertani (LB) media at final concentrations of 0.1 g/l and 1 mM, respectively. The media were then neutralized with KOH. A 0.2-M aqueous solution of *O*-sulfo-L-tyrosine (Watanabe Chemical Industries, Hiroshima, Japan), a 100-mM aqueous solution of N^{ε}-allyloxycarbonyl-L-lysine (AlocLys) (Bachem),

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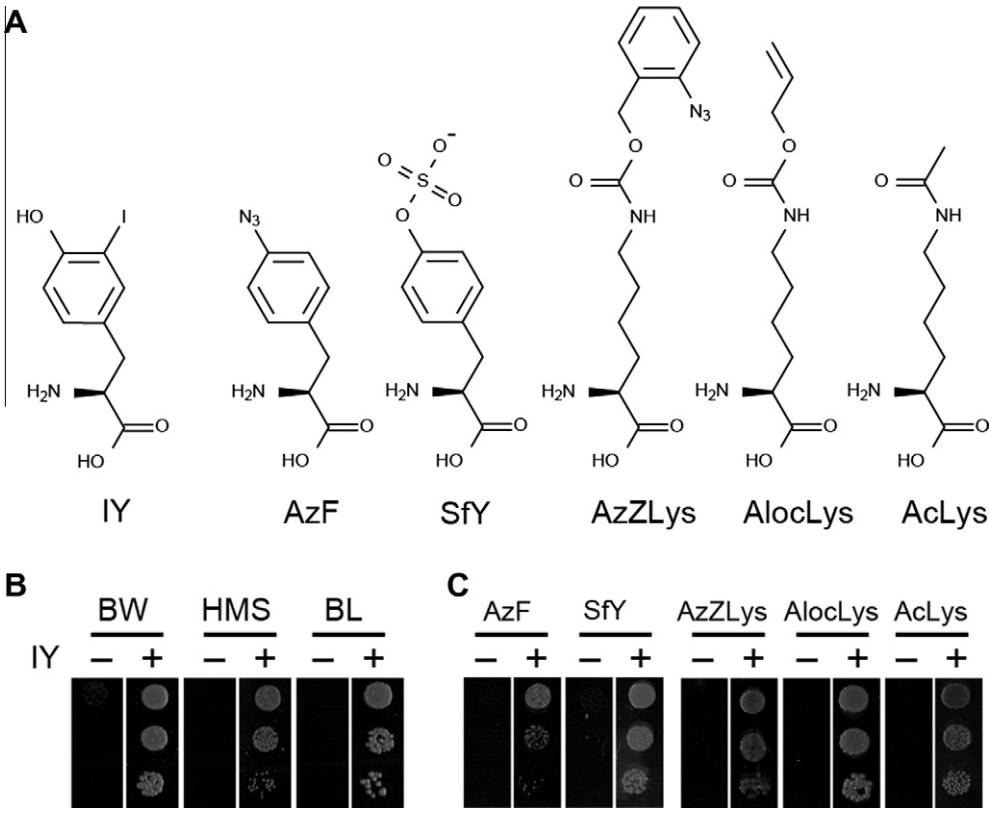

Fig. 1. UAG-codon reassignment in diverse genetic backgrounds and with various non-natural amino acids. (A) Structures of non-natural amino acids used in this study. (B) The growth of the RFzero-iy strains derived from BW25113, HMS174(DE3), and BL21(DE3) was strictly dependent on the supplementation of 3-iodotyrosine in non-selective media. Overnight cell cultures were diluted and spotted on LB plates in the absence (IY−) and presence (IY+) of 3-iodotyrosine. The level of dilution increases from top to bottom. (C) The growth of the RFzero strains with UAG codons completely redefined with the indicated amino acids was strictly dependent on the supplementation of these specific amino acids.

and a 1-M aqueous solution of N^{ε}-acetyl-L-lysine (Bachem) were added at final concentrations of 10, 1, and 10 mM, respectively. N^{ε}-(*o*-azidobenzyloxycarbonyl)-L-lysine (AzZLys) (Shinsei Chemical Co. Ltd., Osaka, Japan) was dissolved in 0.25 N HCl containing 50% (v/v) dimethyl sulfoxide to a concentration of 100 mM, and was added to LB media at a final concentration of 1 mM, which was neutralized with NaOH.

2.2. Plasmids and prfA knockout

The *sucB* ORF engineered to end with UAA was cloned, with its original ribosome-binding sites, downstream of the β-lactamase (*bla*) gene in pBR322, for transcription from the *bla* promoter. To create BAC8, the *sucB* ORF ending with UAA and the upstream chloramphenicol acetyltransferase (*cat*) gene were cloned together downstream of *lolA* in the BAC7 plasmid [5], by homologous recombination using an RT/ET kit (Gene Bridges). *sucB* was expressed from the *cat* promoter. Recombinants with BAC8 were selected against chloramphenicol, at a concentration of 17 μg/ml. The *prfA* knockout was performed in cells transformed with BAC8 and iodoTyrRS-MJR1-gent, which was derived from piodoTyrRS-MJR1-kan [5] by substituting the gentamicin-resistance (*gent*) gene for the kanamycin-resistance (*kan*) gene. A major part of *prfA* was replaced with the Zeocin resistance gene in the chromosome by homologous recombination, as described previously [5]. Cells with knocked-out *prfA* were screened against Zeocin (50–75 μg/ml).

The *tyrS* promoter for expressing the tyrosyl-tRNA synthetase (TyrRS) variants, and the pair of the *lpp* promoter and the *rrnC* terminator for expressing UAG-decoding tRNA were cloned within pBR322 to create pTyr. The archaeal TyrRS specific to 3-iodotyrosine, with Arg in place of Asp286 for enhanced recognition of UAG-decoding tRNAs [6], and the Nap3 $tRNA^{Tyr}_{CUA}$ variant [7] were cloned into pTyr to create pIYN3. Similarly, the pairs of the 4-azidophenylalanine-specific TyrRS variant and the pAzPhe1 tRNA [7] and of the sulfotyrosine-specific TyrRS variant and the Nap3 tRNA were cloned in pTyr to create pAzFA1 and pSfYN3, respectively. RFzero-iy was transformed with these three plasmids, to obtain growth curves and to examine the growth dependence on the specific amino acids by spotting on LB plates. pTacGST [5] carried the glutathione *S*-transferase (*gst*) gene, encoding GST with 25 extra N-terminal amino acids, downstream of the *tac* promoter, and *lacI* or $lacI^q$. The expression units for the three variant-tRNA pairs were cloned in pTacGST to create pTacGST-IYN3, pTacGST-AzFA1, and pTacGST-SfYN3, respectively. pTacGST-IYN3 carried *lacI*, while the other two carried $lacI^q$. RFzero-iy was transformed with these three plasmids to incorporate the specific amino acids into GST in response to UAG. The *gst* gene was engineered to *gst*(3× amb) with UAG codons at positions 25, 47, and 98 (the numbering includes the extra N-terminal amino acids) and *gst*(7× amb) with UAG at these three positions, besides positions 82, 166, 180, and 188. The pPyl plasmid, a derivative of pBR322 with *kan* in place of *bla*, carried two tandem copies of the *Methanosarcina mazei* $tRNA^{Pyl}$ gene, each with the *lpp* promoter and the *rrnC* terminator, and the pair of the *glnŚ* promoter [8] and the *rrnC* terminator for expressing the archaeal pyrrolysyl-tRNA synthetase (PylRS) variants. The PylRS variants recognizing AzZLys and AlocLys [9], and another variant specific for acetyllysine (AcKRS-3) [10], were cloned in pPyl to create pPylAF, pPylF, and pPylAcKR-3, respectively. RFzero-iy was transformed with these three plasmids, to obtain growth curves and to examine the growth dependence on the specific amino acids by spotting on LB plates.

To compare the UAG-decoding efficiencies of the TyrRS variants, a fusion protein between thioredoxin (TRX) and GST with the extra N-terminal peptide was expressed to allow the detection of the truncated product due to aborted translation at the UAG near the N-terminus of GST. The DNA fragment consisting of the TRX and 6× His tags was obtained from pET32b (Novagen) and cloned in pTacGST, to create a fusion protein consisting of TRX, 6× His, and GST in this order, downstream of the *tac* promoter. This fusion gene and *lacI* were then moved to pACYC184 to create pACYC–TRX–GST, which was mutagenized to introduce UAG at position 25 of GST to create pACYC–TRX–GSTam.

2.3. Cell-free protein synthesis

Cell-free coupled transcription/translation was performed essentially as described previously [11]. *M. mazei* $tRNA^{Pyl}$ was prepared by *in vitro* transcription. An acetyllysine-specific variant derived from *M. mazei* PylRS [12] with additional mutations (P5L, N203T, and L301I) was overproduced in *E. coli*. These molecules were added to the reaction to translate UAG to acetyllysine. Nicotinamide and acetyllysine were added at final concentrations of 10 and 20 mM, respectively. A peptide tag (MKDHLIHNHHKHEHAHALVPRGSH) was added to the N-terminal methionine of human histone H4. The protein was produced into the insoluble fraction, and then dissolved with 6 M guanidine hydrochloride and subjected to successive chromatography for purification essentially as described [13,14].

2.4. Mass spectrometry

ESI-MS analyses were commercially performed by Mass Spectrometry Service, Research Resources Center, RIKEN Brain Science Institute (Wako, Japan).

3. Results and discussion

3.1. Engineering of sucB improves the growth of E. coli with UAG reassigned to non-natural amino acid

The lethality of *prfA* knockout has been avoided by expressing UAG-decoding tRNA and introducing seven essential open reading frames (ORFs) engineered to end with another stop codon, UAA, in place of UAG [5], as opposed to erasing more than 300 UAG codons from the chromosome [15]. The identify of UAG corresponds to the amino-acid specificity of the decoding tRNA; in RFzero-iy, which translates UAG to 3-iodo-L-tyrosine, this translation relies on the introduced archaeal pair of UAG-decoding tRNA and the tyrosyl-tRNA synthetase (TyrRS) variant attaching 3-iodotyrosine to this tRNA [16]. Note here that the rapid-codon reassignment left the 300 non-essential genes ending with UAG. We assumed that a few of them might be engineered to end with UAA, like the seven essential genes, so as to guarantee their expression and enhance the growth of RFzero-iy. According to a comprehensive genetic study (Genobase, http://sal.cs.purdue.edu:8097/GB7/index.jsp), thirteen ORFs (*atpE*, *fimB*, *fliNPQE*, *nanC*, *otsA*, *sucB*, *tatD*, *ubiF*, *ulaF*, and *yajL*) are contributing to vigorous growth. These genes were engineered to end with UAA and then separately introduced into RFzero-iy. We found that the engineered *sucB* significantly improved the growth of HST08-based RFzero-iy (Supplementary Fig. S1A); *sucB* is involved in energy regeneration and ends with two successive UAG codons. This finding may explain the poor growth of RFzero-iy, which contrasted with the vigorous growth of RFzero-q that translates UAG as glutamine. In RFzero-q, the endogenous amber suppressor $tRNA^{Gln}$ efficiently decodes UAG, probably allowing the expression of *sucB* to some level, although a C-terminal extra peptide was added to the product due to the readthrough at UAG. On the other hand, the exogenous tRNA-TyrRS pair specific to 3-iodotyrosine does not translate UAG as efficiently as the suppressor $tRNA^{Gln}$, and may cause ribosome to stall at the double UAG site, thus preventing the expression of *sucB*.

3.2. UAG reassignment in diverse genetic backgrounds

The finding with *sucB* showed that the proteome altered by the incorporation of 3-iodotyrosine at the UAG codons, left intact at the ends of 300 genes, can support vigorous growth of the HTS08-based RFzero. We exploited this finding to reassign UAG in diverse genetic backgrounds, other than that of HST08, and to various non-natural amino acids. First, we tried to create RFzero-iy, based on two K-12 strains [BW25113 and HMS174(DE3)] and a B strain [BL21(DE3)], by introducing a plasmid carrying the engineered *sucB* ORF, together with the previously engineered seven ORFs, and another plasmid expressing the archaeal tRNA–TyrRS pair specific to 3-iodotyrosine. The *prfA* gene was successfully knocked out in all of these strains, by replacing it with a marker gene in the chromosome (Supplementary Fig. S2). The resulting BW25113- and BL21-based RFzero-iy strains grew nearly as fast as their *prfA* + parent strains, while the HMS174-based strain grew only moderately slower than its parent (Supplementary Fig. S1B–D). The growth of all of these RFzero-iy strains strictly depended on the supplementation of 3-iodotyrosine in non-selective media (Fig. 1B), which is consistent with the requirement of the cellular UAG-translating activity for avoiding the lethality of *prfA* knockout.

3.3. UAG reassignment to various non-natural amino acids

Next, we redefined UAG with various tyrosine and lysine derivatives (Fig. 1A). The archaeal TyrRS and pyrrolysyl-tRNA synthetase (PylRS) have been engineered to attach these amino acids to UAG-decoding tRNAs in *E. coli*. The TyrRS variants for 4-azidophenylalanine [17] and *O*-sulfotyrosine [18], and the PylRS variants for N^{ε}-acetyllysine [10], N^{ε}-allyloxycarbonyllysine (AlocLys) [9], and N^{ε}-(*o*-azidobenzyloxycarbonyl)-lysine (AzZLys) [9] were expressed in RFzero-iy strain, together with their partner tRNA species decoding UAG. As the endogenous system of RFzero-iy for translating UAG to 3-iodotyrosine does not work in the absence of this amino acid, the introduction of another tRNA-variant pair and the supplementation of its specific amino acid in the media should redefine UAG from 3-iodotyrosine to this amino acid. In fact, the RFzero-iy strains harboring the tRNA-variant pairs specific to 4-azidophenylalanine, *O*-sulfotyrosine, and AlocLys could grow nearly as fast as the parent BW25113 in the absence of 3-iodotyrosine, when the specific amino acids were supplemented in the media (Supplementary Fig. S1E and F). The other RFzero-iy strains, harboring the pairs for AzZLys and acetyllysine, grew moderately slower than BW25113 (Supplementary Fig. S1F). The growth of the created RFzero strains strictly depended on the supplemented, specific non-natural amino acids (Fig. 1C), indicating that the UAG identity had switched from
3-iodotyrosine to these amino acids. These results showed that the rapid codon-reassignment is applicable with diverse genetic backgrounds and various non-natural amino acids. The growth of the UAG-reassigned strains was sufficiently fast for large-scale protein production.

3.4. Protein synthesis with non-natural amino acids at multiple defined sites

To demonstrate protein synthesis with non-natural amino acids in RFzero strains, we replaced some of the 15 tyrosine codons in the glutathione *S*-transferase (GST) gene with UAG; we replaced

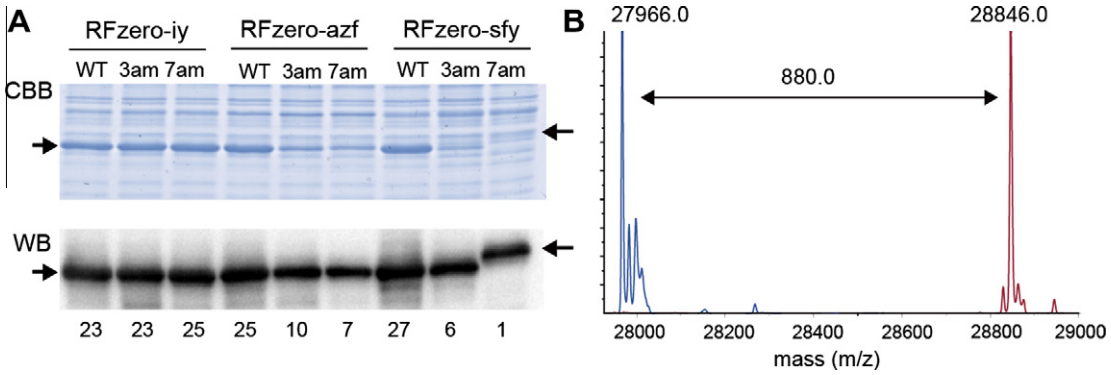

Fig. 2. Biosynthesis of GST variants with three and seven tyrosine derivatives. (A) The arrows indicate the positions corresponding to the full-length GST products in the gel stained with blue dye (marked with "CBB") and the western-blotting (WB) using the anti-GST antibody. The wild-type *gst*, *gst*(3× amb), and *gst*(7× amb) genes (lanes labeled with "WT", "3am", and "7am", respectively) were introduced into the indicated strains. The numbers below each lane show the estimated yields (mg/l culture). (B) ESI-MS analyses of the wild-type GST (blue line) and its variant with seven iodotyrosines (red line) with predicted masses of 27,966.2 and 28,847.5 Da, respectively. The difference corresponds to the mass of seven iodine atoms, less that of seven hydrogen atoms (881.3 Da).

three and seven tyrosine codons encoding the residues on the protein surface by UAG, to create the *gst*(3× amb) and *gst*(7× amb) variant genes, respectively, with the remaining tyrosine codons left intact. These variant genes were introduced into the BW2115-based RFzero strains translating UAG to 3-iodotyrosine, 4-azidophenylalanine, and *O*-sulfotyrosine (RFzero-iy, -azf, and -sfy, respectively), and were found to express the full-length products in all of these strains (Fig. 2A). RFzero-iy synthesized the variants with three and seven 3-iodotyrosines in almost equal amounts as the wild-type GST; the incorporation of seven iodotyrosines in the *gst*(7× amb) product was confirmed by mass spectrometry (Fig. 2B). Although the variant yields were reduced in RFzero-azf and RFzero-sfy, even the variant with seven *O*-sulfotyrosines, with the lowest yield, was produced in a milligram-order amount per liter of cell culture. The reduced yields suggested that the rates of protein synthesis with 4-azidophenylalanine and *O*-sulfotyrosine are slower than that with 3-iodotyrosine, probably because the translation machinery or the assimilation mechanism works on various non-natural amino acids with different efficiencies [19]. In fact, the two tyrosine derivatives were also incorporated with significantly lower efficiencies than 3-iodotyrosine in a *prfA* + background (Supplementary Fig. S3). The yield of a variant could also be reduced, if the incorporation of non-natural amino acids at more sites increasingly destabilizes the protein.

3.5. Production of histone H4 fully acetylated at four specific sites

Finally, we produced histone H4 with acetyllysines at specific multiple sites, by taking advantage of the lineup of RFzero with diverse genetic backgrounds. Histone H4 has four lysines at positions 5, 8, 12 and 16 in the N-terminal peptide as the major post-translational acetylation sites. A method to synthesize homogenous histones modified at certain positions will be useful for epigenetics studies and developing therapeutic agents acting on chromatin remodeling. Cell-free synthesis should serve this purpose well, because nicotinamide can be directly added at a high concentration to the cell extract to prevent deacetylation, which causes heterogeneity. We used BL21-based RFzero-iy to prepare the cell extract, since the BL21 extract was reportedly highly productive [11]. The extract was dialyzed to remove 3-iodotyrosine, and then was supplemented with the PylRS variant specific to acetyllysine and the partner tRNA decoding UAG. The histone-H4 mutant gene, with all four of the acetylation sites encoded by UAG, successfully produced the full-length histone H4 with a yield of 0.2 mg per ml-reaction; a mass spectrometric analysis confirmed the incorporation of the four acetyllysines (Fig. 3).

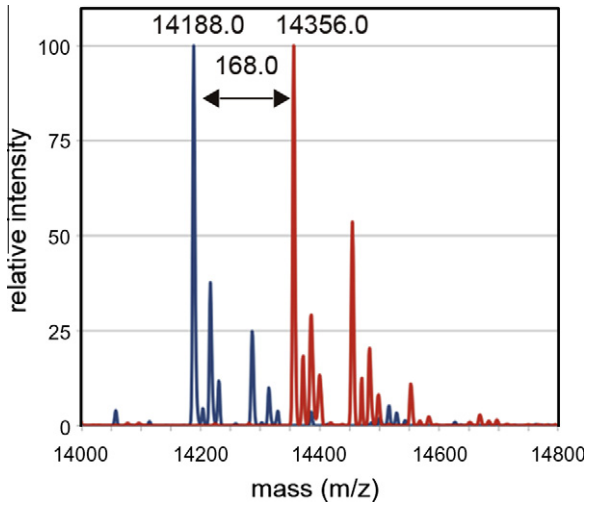

Fig. 3. ESI-MS analyses of histones H4 with four acetyllysines at specific sites (red line) and with no acetylation (blue line). The predicted mass for the unmodified molecule is 14,188.8 Da, and the difference of 168.0 Da corresponds to the addition of four acetyl groups (168.1 Da).

4. Concluding remarks

Non-natural amino acids have been genetically encoded in living cells, with codons retaining their original identities [2–4]. This ambiguous codon assignment has limited the availability of canonical amino acids or the number of incorporation sites for non-natural amino acids in one protein. The present study showed that codon reassignment, free from these drawbacks, is a promising method to synthesize modified proteins. Our finding that UAG can be rapidly reassigned by replacing it at the ends of a small number of UAG-ending ORFs, as opposed to erasing all of the genomic UAG codons, might be helpful for planning further reassignment of synonymous codons, to encode a variety of non-natural amino acids in the genetic code.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.020.

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