

Tunable translational control using site-specific unnatural amino acid incorporation in *Escherichia coli*

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ABSTRACT

Translation of target gene transcripts in *Escherichia coli* harboring UAG amber stop codons can be switched on by the amber-codon-specific incorporation of an exogenously supplied unnatural amino acid, 3-iodo-L-tyrosine. Here, we report that this translational switch can control the translational efficiency at any intermediate magnitude by adjustment of the 3-iodo-L-tyrosine concentration in the medium, as a tunable translational controller. The translational efficiency of a target gene reached maximum levels with 10^{-5} M 3-iodo-L-tyrosine, and intermediate levels were observed with suboptimal concentrations (approximately spanning a 2-log_{10} concentration range, 10^{-7} – 10^{-5} M). Such intermediate-level expression was also confirmed in individual bacteria.

Subjects Bioengineering, Biotechnology, Microbiology

Keywords Translational regulation, *Escherichia coli*, Expression system

INTRODUCTION

Controlling gene expression is a key methodology for biotechnology. Particularly, its fine-tuning and optimization are important for construction of artificial gene circuits in synthetic biology and for metabolic engineering. Tunable conditional expression systems regulated by extracellular inducers/repressors are thus very useful.

We previously demonstrated a translational switch using site-specific unnatural amino acid (UAA) 3-iodo-L-tyrosine (IY) incorporation in natural amber suppressor-free strains of *Escherichia coli* (Minaba & Kato, 2014). Although IY is not perfectly “unnatural” and found in very specific biological tissues such as the thyroid cells (Tietze *et al.*, 1989) and the sponge skeletons (Ueberlein *et al.*, 2014), we cannot detect IY ubiquitously in natural environments. The translational switch is based on conditional read-through of the UAG amber stop codons that are inserted in target genes (Fig. 1). A variant of aminoacyl-tRNA synthetase (aaRS) IYRS that was derived from the archaeobacterium *Methanocaldococcus jannaschii* specifically recognizes both IY and an amber suppressor tRNA (tRNA_{CUA}) MJR1 (Sakamoto *et al.*, 2009). Extracellular IY is taken up and incorporated into proteins at sites of the amber codons in the IYRS/MJR1-expressing cells. The target gene transcripts with the amber stop codons inserted next to the AUG translational start codon are translated only in the presence of IY. The absence of IY prevents translation of the target genes.

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Additional Information and
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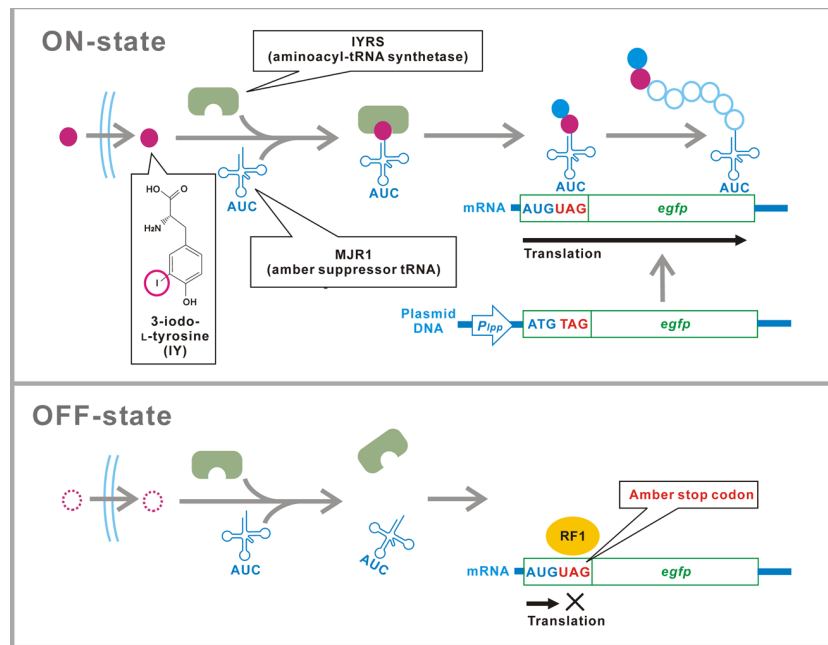


Figure 1 Schematic of the translational switch using the amber codon-specific IY. An amber stop codon is inserted next to the ATG translational start codon in the target gene (*egfp*). MJR1 is an amber suppressor tRNA. IYRS is an aminoacyl-tRNA synthetase that orthogonally recognizes IY and MJR1. Extracellular IY is taken up by the bacteria. The addition of IY in the media results in amber stop codon read-through and translation of the target gene. Translation is interrupted in the absence of IY. RF1, peptide chain release factor 1.

The UAA-controlling translational switch has distinct features for gene regulation (Minaba & Kato, 2014). First, this translational switch also can be controlled by the induction/repression of either aaRS or tRNA_{CUA}, in addition to the presence/absence of UAA. The switchability can be modulated by combination use with aaRS- and/or tRNA_{CUA}- switching. Second, UAA does not naturally exist. Third, UAA is a “building block” that forms an aminoacyl-tRNA and target proteins, distinct from simple regulation-specific molecules, such as isopropyl β-D-1-thiogalactopyranoside (IPTG) for the derepression of the lactose operator. The switching mechanism involves the direct incorporation of UAA into target proteins, and is not effective for interventional systems. The second and third characteristics indicate that the UAA-controlling translational switch is robust against environmental and host-endogenous noises. Although an inducible tRNA_{CUA} has been used as a similar translational switch based on amber suppression, these features are not found (Zengel & Lindahl, 1981; Herring, Glasner & Blattner, 2003). Fourth, the UAA-controlling translational switch can be used in combination with any established transcriptional switches to obtain a synergistic regulatory effect. Using this strategy, we constructed a high-yield and zero-leakage expression system, in which strong expression by the T7 promoter was maintained under the induction condition, and almost no proteins were expressed under the repression condition by double control of transcription-translation.

Riboswitches and small regulatory RNAs (sRNAs) are well known as post-transcriptional controlling tools in *E. coli*. Some riboswitches that are located in non-coding portions of mRNAs can regulate gene expression *in cis* by binding a specific small molecule via controlling translational initiation and/or mRNA degradation (Caron *et al.*, 2012). The sRNAs are trans-acting and target gene alterations are not required, although off-target effects are often detected (Bobrovskyy & Vanderpool, 2013). The sRNAs modulate the translation via controlling translation initiation and/or mRNA degradation. The distinct characteristics of these RNA-based post-transcriptional switches suggest that the UAA-controlling translational switch is complementary rather than competitive with the others.

To date, many systems for the incorporation of various UAAs have been developed (Liu & Schultz, 2010). In addition, similar translational switches controlled by UAAs are expected to be applicable also for eukaryotes, such as yeasts, nematodes, insects, mammalian cells, and plants, because the site-specific unnatural amino acid incorporation systems have already been introduced (Chin *et al.*, 2003; Greiss & Chin, 2011; Bianco *et al.*, 2012; Sakamoto *et al.*, 2002; Li *et al.*, 2013).

The target gene products that are controlled by the UAA-controlling translational switch necessitate the incorporation of UAA. Although the UAA-incorporation may cause functional alterations in some target proteins, we can avoid this problem by neutral site selection, incorporation into tag or processed-out sequences. Alternatively, the UAAs can be used as tools to facilitate purification or tracking of target gene products (Minaba & Kato, 2014).

In previous studies, we only focused on the on-off aspect of the IY-controlling translational switch. Here, we studied the intermediate states between the fully on- and off-states of the translational switch. The results suggest that this switch can control the translational efficiency at any intermediate magnitude by adjustment of the appropriate IY concentration, and thus, function as a tunable translational controller.

MATERIALS AND METHODS

Fluorescence measurement of pooled bacteria

Assays were performed as described previously (Minaba & Kato, 2014). Briefly, we used *E. coli* BL21-AI (F^- *ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) araB::T7RNAP-tetA*) carrying the plasmid pTYR MjIYRS2-1(D286) MJR1 × 3 encoding IYRS and MJR1 and the amber-inserted EGFP expression plasmid (driven by the *E. coli* *lpp* promoter with an amber stop codon TAG inserted next to the start codon ATG). The sequence for the EGFP expression plasmid is shown in Fig. S1. An overnight (approximately 16 h) culture of bacteria was resuspended in an equal volume of liquid LB medium (1% bacto tryptone, 0.5% yeast extract, and 1% NaCl) containing various concentrations of optically-pure IY. IY was directly dissolved and diluted in LB medium. After a 6 h culture (2 ml in a 14 ml-culture tube at 37 °C with rotary shaking at 200 rpm), the bacteria were collected by centrifugation (1,800 × *g* for 3 min). The pellet was washed and was resuspended in an equal volume of 0.9% (wt/vol) NaCl, and this wash step was repeated twice. An aliquot of

the bacterial suspension (150 μ l) was diluted in 3.0 ml of the 0.9% NaCl, and the OD₅₉₀ was measured. The fluorescence intensity of the bacterial population was measured using a Shimadzu RF-5300PC spectrofluorometer (excitation, 480 nm; emission, 515 nm). The background fluorescence was estimated using the bacteria carrying a MJR1-deleted plasmid (Δ MJR1) at IY = 0 (no significant IY-dependency was observed, Fig. S2). The background-subtracted values were used to calculate the points for the dose-response curve. For the time course measurement of EGFP accumulation, we cultured the bacteria in 20 ml LB medium in a 200 ml culture flask. Aliquots of the bacterial culture (150 μ l) were withdrawn at various time points, and the OD₅₉₀ and fluorescence were measured.

Photomicrographs

Photomicrographs and Nomarski differential interference contrast images of the fluorescent bacteria were recorded using both a Carl Zeiss Axioskop 2 with a 38-HE Endow GFP filter-set (Carl Zeiss, Jena, Germany) and a Roper Scientific Photometrics CoolSNAP ver.1.1 (Roper Industries, Sarasota, Florida, USA).

Image analyses

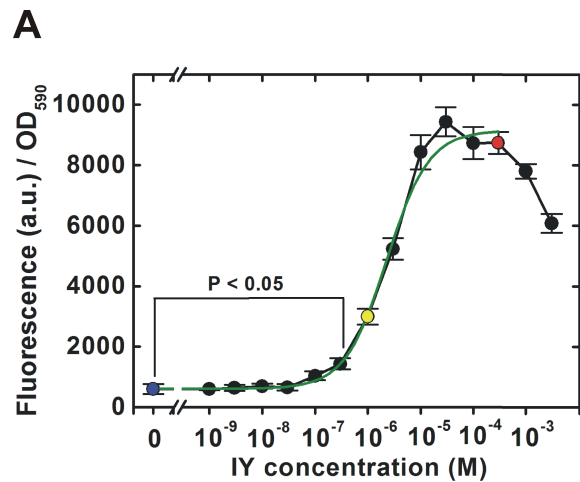
Analyses of the fluorescence images were performed using ImageJ 1.48v (National Institutes of Health, Bethesda, Maryland, USA). Prior to analyses, the fluorescence images were confirmed as not being saturated at any pixels. Three-dimensional graphs of the intensities of the pixels were generated using Surface Plot command. The fluorescence intensity of individual bacteria was measured using Particle Analysis command. A background value (bacteria-absent area) was used as a threshold for particle detection. A range of particle area was set to detect only individual and not-overlapping bacterial cell images.

Growth curve

Growth curves were determined as previously described with some modifications (Minaba & Kato, 2014). Overnight cultures of bacteria were diluted (1:200) in fresh LB medium and were incubated at 37 °C with rotary shaking at 200 rpm. After reaching a visible density (around OD₅₉₀ = 0.1), OD₅₉₀ was measured every 20 min for 2 h.

RESULTS AND DISCUSSION

To characterize the intermediate states of the translational switch using the IY-incorporation system, the IY dose-dependency of translational efficiency for a target gene was first determined for a bacterial population (Fig. 2A). As shown in Fig. 1, an EGFP gene containing an amber stop codon next to the ATG translational start codon was constitutively transcribed by the *lpp* promoter in the *E. coli* BL21-AI strain expressing IYRS and MJR1. The translational efficiency was estimated from the EGFP fluorescence 6 h after IY addition into the medium. The “gross” translational efficiency for the population started to increase significantly at 3×10^{-7} M and reached maximum levels at 10^{-5} M. Intermediate levels of translational efficiency were observed at a 2-log₁₀ suboptimal concentration of IY (10^{-7} – 10^{-5} M), suggesting that the translational efficiency can be tuned in this concentration range. The 50% effective concentration was estimated to be



Model: Logistic

$$F = F_{\max} + \frac{F_{\min} - F_{\max}}{1 + (C_{IY} / C_0)^p}$$

$$F_{\max} = (9.15 \pm 0.29) \times 10^3$$

$$F_{\min} = (0.61 \pm 0.03) \times 10^3$$

$$C_0 = (2.30 \pm 0.31) \times 10^{-6} \text{ M}$$

$$p = 1.10 \pm 0.11$$

Goodness-of-fit:

$$\chi^2 / \text{DoF} = 0.93$$

$$R^2 = 0.99$$

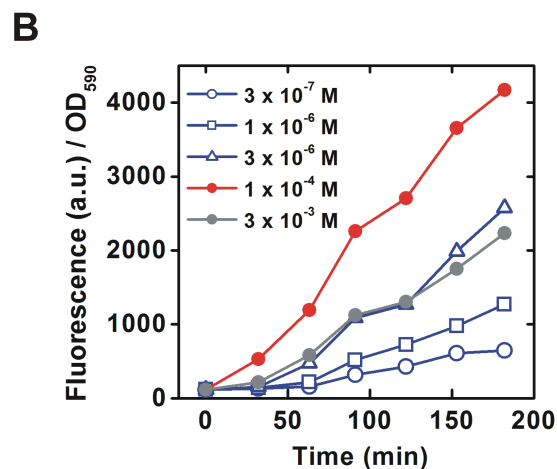


Figure 2 IY dose-dependent change in translational efficiency for a bacterial population. The experimental system is schematically shown in Fig. 1 (A) Dose-response curve. Bacteria were cultured in media containing various concentrations of IY for 6 h. Colored circles indicate the samples for Fig. 3. Data are shown as mean \pm SEM. $n = 3$ independent experiments using completely separated bacterial cultures. Statistical analysis was performed using Welch's t -test in Excel ver. 14.0. (continued on next page...)

Figure 2 (...continued)

A single fitted dose-response curve (log-logistic, $IY = 0-3 \times 10^{-4}$ M) was generated using Origin7. The equation for the fitted curve and assessments of goodness-of-fit are represented under the graph. F , fluorescent intensity (arbitrary unit); F_{\max} , the initial F value (right horizontal asymptote); F_{\min} , the final F value (left horizontal asymptote); C_{IY} , IY concentration (M); C_0 , 50% effective C_{IY} (point of inflection); χ^2 , reduced chi-squared value; DoF, degrees of freedom. (B) Time course of EGFP accumulation.

3×10^{-6} M. The IY concentration-dependent change of the gross translational efficiency was also confirmed by a direct measurement of the time course for EGFP accumulation (Fig. 2B). The EGFP accumulation rate was clearly slower at suboptimal concentrations than that at the optimal concentration. The accumulation rate increased with increasing IY concentration, also suggesting that an intermediate translational efficiency could be obtained in the suboptimal concentration range.

Intermediate levels of gene expression for the pooled bacteria are not always equal to that for an individual bacterium. In the case of conditional gene expression by the *araBAD* promoter, intermediate expression levels in the cultures reflected a population average of the induced and non-induced cells, i.e., each cell responded to an inducer L -arabinose at a suboptimal concentration in an all-or-none manner (Siegele & Hu, 1997; Guzman et al., 1995). A similar non-uniform induction was also reported for the *lac* operon (Novick & Weiner, 1957; Maloney & Rotman, 1973). We therefore determined whether the IY dose-dependent change of EGFP fluorescence in individual cells using fluorescence images (Fig. 3). The fluorescence intensity of these images was quantified by image analysis, and spatial maps were generated. The fluorescence intensity clearly increased with increasing IY concentration. Fluorescent intensity distribution histograms of individual bacteria were also examined. As expected from the fluorescence images and their spatial maps, the peak frequency at a suboptimal IY concentration (1×10^{-6} M) was located between those at zero and the saturation concentration, indicating that the translational efficiency of individual bacteria can be controlled at intermediate values. This response is unlike the mix-population of all-or-none responding cells observed in a suboptimal concentration of inducers for both the *araBAD* promoter and the *lac* operon. The frequency distribution was relatively wide and overlapped with those at zero and at the saturation concentration, suggesting that the responses of individual cells are variable at suboptimal concentrations. The all-or-none behavior of the *lac* operon was explained by an autocatalytic positive feedback loop that led to a burst of synthesis of galactoside permease LacY (Novick & Weiner, 1957; Maloney & Rotman, 1973). A similar model was also proposed for the *araBAD* promoter system (Siegele & Hu, 1997). In these models, the intracellular concentration of either lactose or arabinose tends to be either very low or saturated. To obtain a better linear response to an extracellular inducer concentration, IPTG is used instead of lactose in a $lacY^-$ strain for control of the *lac* promoter (Khlebnikov & Keasling, 2002). Similarly, mutant strains in which arabinose transport and degradation genes are deficient can avoid the all-or-none response of the *araBAD* promoter (Bowers et al., 2004). These modifications impair the positive feedback loop and confer linearity between extracellular and intracellular inducer concentrations. In the case of the IY-controlling

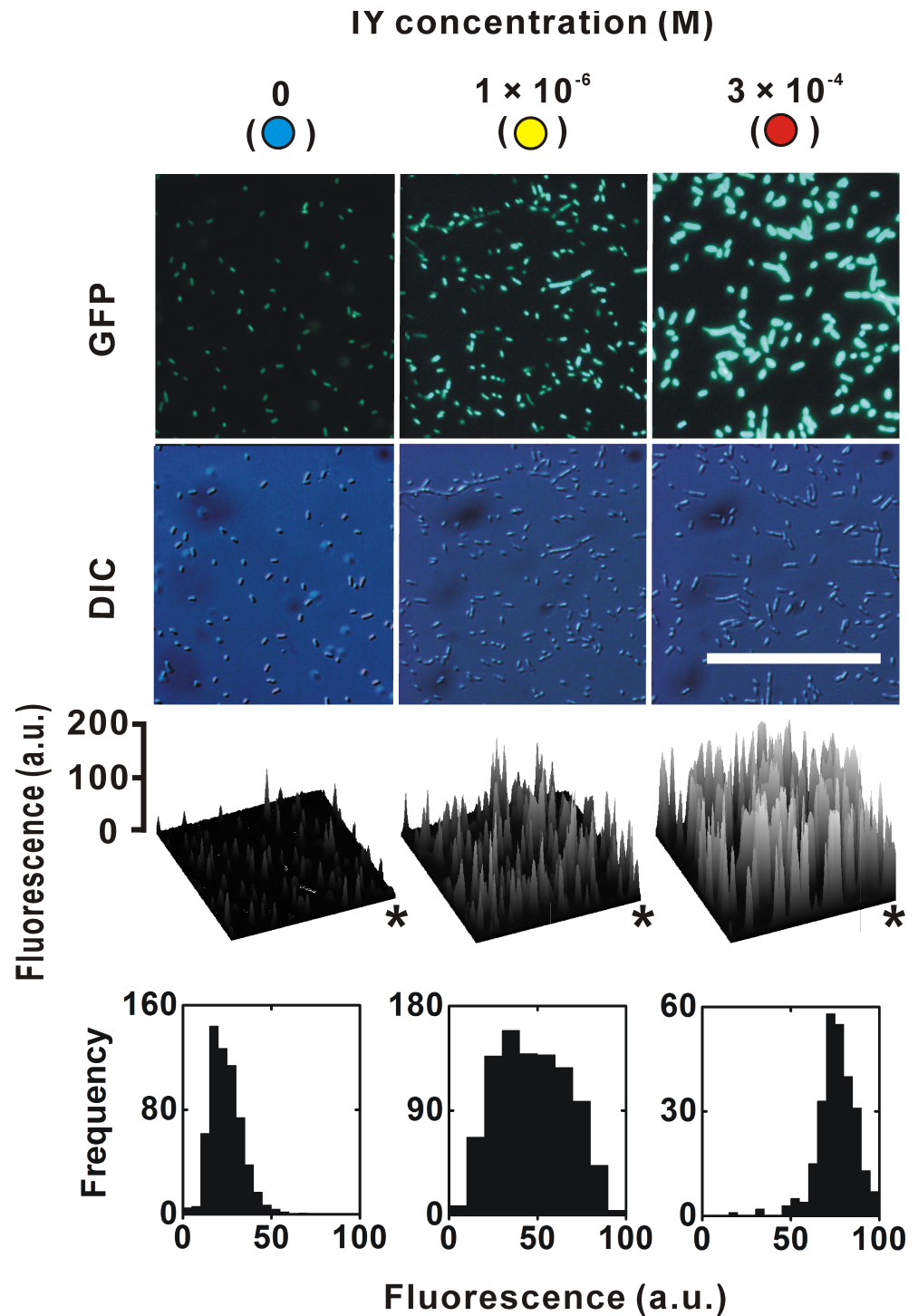


Figure 3 EGFP expression in individual bacteria. The sampling points are indicated in the dose-response curve in Fig. 2A. Upper and lower photographs are epifluorescence images and Nomarski differential interference contrast images, respectively. The photographic conditions were constant for all of the fluorescence images. Calibration bar = 100 μm . Asterisks in spatial distribution graphs indicate the upper right corners of the fluorescence images. The frequency in the histograms indicates the number of individual bacteria.

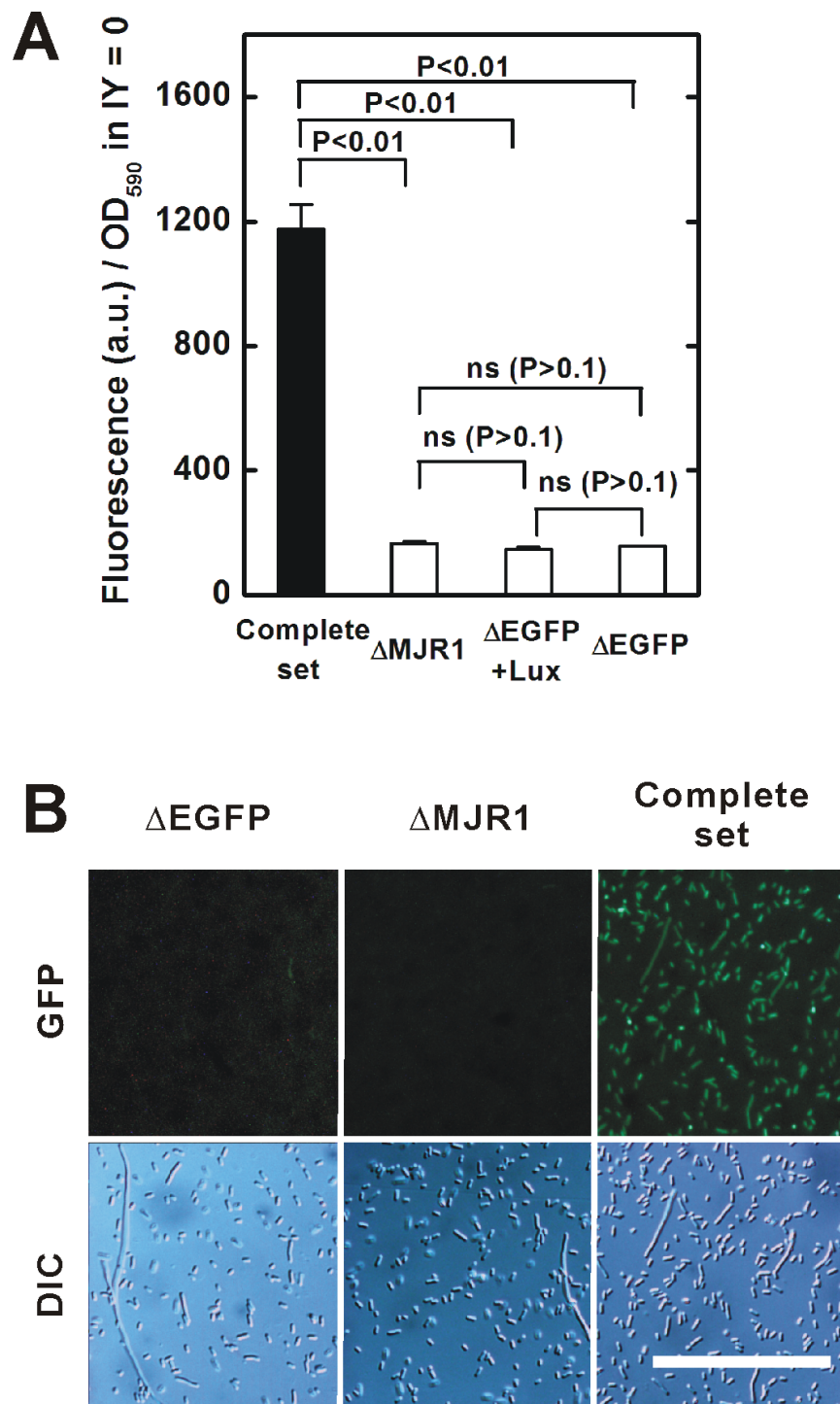


Figure 4 Leaky translation in the absence of IY. (A) Leaky translation for a bacterial population. Note that the measured fluorescence contains both EGFP-fluorescence and non-EGFP background. Complete set, the strain carrying the plasmid pTYR MjIYRS2-1(D286) MJR1 × 3 and the amber-inserted EGFP expression plasmid (driven by the *E. coli lpp* promoter); ΔMJR1, a derivative strain carrying a MJR1-deleted plasmid; ΔEGFP, a derivative strain lacking the (continued on next page...)

Figure 4 (...continued)

amber-inserted EGFP gene expression plasmid; Δ EGFP + Lux, a derivative strain in which the amber-inserted EGFP gene was substituted with an amber-inserted LuxB gene from the bacterium *Vibrio harveyi* as a vector control (Schultz & Yarus, 1990). Data are shown as mean \pm SEM. $n = 3$ independent experiments. Statistical analysis was performed using Welch's t -test. ns, not significant. (B) Observation of leaky translation in individual bacteria. The EGFP fluorescence was measured in the absence of IY. In these images, non-specific background fluorescence was completely filtered out (note that this method is distinct from that of Fig. 4A). The exposure time for the EGFP images was twice that in Fig. 3.

translational switch, the velocity of translation by a suppressor IY-tRNA is expected to depend on the intracellular IY concentration if the concentrations of the suppressor tRNA, IYRS, and peptide release factors are constant (Yarus *et al.*, 1986). The intracellular IY concentration was possibly at a subsaturation level at the suboptimal concentration of extracellular IY. Although the uptake mechanism of IY remains unclear, positive feedback loops as seen in the *lac* operon or the *araBAD* system may be weak or not be involved (Pittard, 1996).

Approximately 7% of the maximum translation was detected even in the absence of IY, as also described previously (Minaba & Kato, 2014). The leaky translation was completely abolished by deletion of the MJR1 gene, suggesting that mischarges of MJR1 were the cause (Figs. 4A and 4B). Although some countermeasure techniques have been proposed, reduction of leaky translation needs to be a priority (Minaba & Kato, 2014).

The translational efficiency decreased from the saturation level at an extremely high IY concentration (Figs. 2A and 2B). Although its mechanism remains to be elucidated, the decrease may not be due to non-specific toxicity of IY because the bacterial growth rates did not decrease both in the IY-incorporating strains and in the parent strain (Fig. S3).

In this study, we evaluated the IY-controlling translational switch using a single setting (a single amber codon in one position, and the single target gene EGFP). In the future, further studies using this application should clarify how general this system is.

CONCLUSIONS

The translational switch using site-specific IY incorporation can be used as a “tunable translational controller” that regulates the translational efficiency in each individual cell. Using this controller, we expect to be able regulate the translational efficiency over a wide range in combination with any transcriptional controlling systems (Minaba & Kato, 2014). The tunable translational controller is a promising tool for conditional fine-tuning and for optimizing the construction of artificial gene circuits in synthetic biology and in metabolic engineering (Yadav *et al.*, 2012).

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The author declares there is no competing interests.

Author Contributions

- Yusuke Kato conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Supplemental Information

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REFERENCES

- Bianco A, Townsley FM, Greiss S, Lang K, Chin JW. 2012.** Expanding the genetic code of *Drosophila melanogaster*. *Nature Chemical Biology* **8**:748–750 DOI [10.1038/nchembio.1043](https://doi.org/10.1038/nchembio.1043).
- Bobrovskyy M, Vanderpool CK. 2013.** Regulation of bacterial metabolism by small RNAs using diverse mechanisms. *Annual Review of Genetics* **47**:209–232 DOI [10.1146/annurev-genet-111212-133445](https://doi.org/10.1146/annurev-genet-111212-133445).
- Bowers LM, LaPoint K, Anthony L, Pluciennik A, Filutowicz M. 2004.** Bacterial expression system with tightly regulated gene expression and plasmid copy number. *Gene* **340**:11–18 DOI [10.1016/j.gene.2004.06.012](https://doi.org/10.1016/j.gene.2004.06.012).
- Caron MP, Bastet L, Lussier A, Simoneau-Roy M, Massé E, Lafontaine DA. 2012.** Dual-acting riboswitch control of translation initiation and mRNA decay. *Proceedings of the National Academy of Sciences of the United States of America* **109**:E3444–E3453 DOI [10.1073/pnas.1214024109](https://doi.org/10.1073/pnas.1214024109).
- Chin JW, Cropp A, Anderson C, Mukherji M, Zhang Z, Schulz PG. 2003.** An expanded eukaryotic genetic code. *Science* **301**:964–967 DOI [10.1126/science.1084772](https://doi.org/10.1126/science.1084772).
- Greiss S, Chin JW. 2011.** Expanding the genetic code of an animal. *Journal of the American Chemical Society* **133**:14196–14199 DOI [10.1021/ja2054034](https://doi.org/10.1021/ja2054034).
- Guzman LM, Belin D, Carson MJ, Beckwith J. 1995.** Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *Journal of Bacteriology* **177**(14):4121–4130.

- Herring CD, Glasner JD, Blattner FR. 2003. Gene replacement without selection: regulated suppression of amber mutations in *Escherichia coli*. *Gene* 331:153–163 DOI 10.1016/S0378-1119(03)00585-7.
- Khlebnikov A, Keasling JD. 2002. Effect of lacY expression on homogeneity of induction from the P(tac) and P(trc) promoters by natural and synthetic inducers. *Biotechnology Progress* 18:672–674 DOI 10.1021/bp010141k.
- Li F, Zhang H, Sun Y, Pan Y, Zhou J, Wang J. 2013. Expanding the genetic code for photoclick chemistry in *E. coli*, mammalian cells, and *A. thaliana*. *Angewandte Chemie International Edition* 52:9700–9704 DOI 10.1002/anie.201303477.
- Liu CC, Schultz PG. 2010. Adding new chemistries to the genetic code. *Annual Review of Biochemistry* 79:413–444 DOI 10.1146/annurev.biochem.052308.105824.
- Maloney PC, Rotman B. 1973. Distribution of suboptimally induced β -D-Galactosidase in *Escherichia coli*: the enzyme content of individual cells. *Journal of Molecular Biology* 73(1):77–91 DOI 10.1016/0022-2836(73)90160-5.
- Minaba M, Kato Y. 2014. High-yield, zero-leakage expression system with a translational switch using site-specific unnatural amino acid incorporation. *Applied and Environmental Microbiology* 80(5):1718–1725 DOI 10.1128/AEM.03417-13.
- Novick A, Weiner M. 1957. Enzyme induction as an all-or-none phenomenon. *Proceedings of the National Academy of Sciences of the United States of America* 43(7):553–566 DOI 10.1073/pnas.43.7.553.
- Pittard AJ. 1996. Biosynthesis of the aromatic amino acids. In: Neidhardt FC, Durtiss R, Ingraham L, Lin CC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE, eds. *Escherichia coli and Salmonella: cellular and molecular biology*. 2nd edition. Washington D.C.: ASM Press, 458–484.
- Sakamoto K, Hayashi A, Sakamoto A, Kiga D, Nakayama H, Soma A, Kobayashi T, Kitabatake M, Takio K, Saito K, Shirouzu M, Hirao I, Yokoyama S. 2002. Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. *Nucleic Acids Research* 30:4692–4699 DOI 10.1093/nar/gkf589.
- Sakamoto K, Murayama K, Oki K, Iraha F, Kato-Murayama M, Takahashi M, Ohtake K, Kobayashi T, Kuramitsu S, Shirouzu M, Yokoyama S. 2009. Genetic encoding of 3-iodo-L-tyrosine in *Escherichia coli* for single-wavelength anomalous dispersion phasing in protein crystallography. *Structure* 17(3):335–344 DOI 10.1016/j.str.2009.01.008.
- Schultz DW, Yarus M. 1990. A simple and sensitive *in vivo* luciferase assay for tRNA-mediated nonsense suppression. *Journal of Bacteriology* 172(2):595–602.
- Siegele DA, Hu JC. 1997. Gene expression from plasmids containing the *araBAD* promoter at subsaturating inducer concentrations represents mixed populations. *Proceedings of the National Academy of Sciences of the United States of America* 94(15):8168–8172 DOI 10.1073/pnas.94.15.8168.
- Tietze F, Kohn LD, Kohn AD, Bernardini I, Andersson HC, Adamson MD, Harper GS, Gahl WA. 1989. Carrier-mediated transport of monoiodotyrosine out of thyroid cell lysosomes. *Journal of Biological Chemistry* 264:4762–4765.
- Ueberlein S, Machill S, Niemann H, Proksch P, Brunner E. 2014. The skeletal amino acid composition of the marine demosponge *Aplysina cavernicola*. *Marine Drugs* 12:4417–4438 DOI 10.3390/md12084417.

- Yadav VG, Mey MD, Lim CG, Ajikumar PK, Stephanopoulos G. 2012.** The future of metabolic engineering and synthetic biology: towards a systematic practice. *Metabolic Engineering* **14**(3):233–241 DOI [10.1016/j.ymben.2012.02.001](https://doi.org/10.1016/j.ymben.2012.02.001).
- Yarus M, Cline SW, Wier P, Breeden L, Thompson RC. 1986.** Actions of the anticodon arm in translation on the phenotypes of RNA Mutants. *Journal of Molecular Biology* **192**:235–255 DOI [10.1016/0022-2836\(86\)90362-1](https://doi.org/10.1016/0022-2836(86)90362-1).
- Zengel JM, Lindahl L. 1981.** High-efficiency, temperature-sensitive suppression of amber mutations in *Escherichia coli*. *Journal of Bacteriology* **145**:459–465.