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Translation Enhancement by a Short Nucleotide Insertion at 5′UTR: Application to an In Vitro Cell-Free System and a Photosynthetic Bacterium

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Abstract: We previously showed that insertion of *Dictyostelium* gene sequences, such as *mlcR*, upstream of the Shine–Dalgarno sequence, positively impacts downstream gene expression in *Escherichia coli*. However, the mechanism by which protein production is facilitated and its applicability to other bacteria remains unknown. In this study, a translation-enhancing effect, associated with this system, on the mRNA amount and property as well as the versatility of the method has been demonstrated. The insertion of *mlcR*-terminal 25 bp (*mlcR25*) stabilized the mRNAs and led to increased mRNA levels in *E. coli*. In the in vitro translation system, a four-fold enhancement was observed when DNA was used as the template, and a three-fold enhancement was observed when mRNA was used as the template. This suggests that *mlcR25* has an effect on the facilitation of the interaction between mRNA and ribosome. Furthermore, when this enhancement system was adapted to the photosynthetic bacterium *Rhodobacter capsulatus*, a more than six-fold increase in translation was observed. Thus, we propose that enhanced translation by *mlcR25* is mediated by mechanisms that help the translation machinery to work efficiently, and the system can be applied to bacteria other than *E. coli*.

Keywords: *Escherichia coli*; *Rhodobacter capsulatus*; *Dictyostelium discoideum*; translation; protein production

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1. Introduction

Gene expression involves the transcription of mRNA and its translation into proteins. *Escherichia coli*, a Gram-negative γ-proteobacterium, is a model organism for which the mechanism of gene expression has been studied extensively. Important factors that affect transcription include sequence characteristics, such as the promoter, terminator, Shine–Dalgarno (SD) sequence [1], and ribosome-binding region in the 5′- or 3′-untranslated region (UTR). In addition, recent accumulating evidence indicates that mRNA stability and codon usage are important factors for the regulation of gene expression [2–11]. *Escherichia coli* is suitable for producing various functional proteins at a low cost and high yield; thus, methods are being developed to regulate the protein-expression levels [12].

The strength of the aforementioned transcriptional factors varies owing to differences in their sequences. Therefore, while the simplest approach is to identify a promoter with enhanced activation, attempts are also being made to optimize the upstream sequence of the SD sequence [12]. Recently, we reported that the insertion of a gene sequence from the eukaryotic cellular slime mold *Dictyostelium discoideum* (e.g., *mlcR* encoding myosin regulatory light chain) upstream of the SD sequence increased protein production in *E. coli* [13]. We named this phenomenon Translation Enhancement by a *Dictyostelium* gene sequence (TED). TED is a straightforward method used to increase protein expression levels by inserting a short sequence in the 5′-UTR (Figure 1). In this method, the insertion or substitution of the *Dictyostelium* gene sequence into an existing vector or possibly the genome enhances protein synthesis, which leads to an increased yield of the desired

protein. Currently, TED that uses a 25 bp sequence of the 3′-end of *mlcR* from *Dictyostelium* (*mlcR25*) has been the most effective [13]. Replacing the T7 phi10 [14] in the pET vector, a typical vector for protein production, with *mlcR25* can further increase production. An interesting aspect of TED used for *mlcR25* is that sufficient fluorescence emitted from the green fluorescent protein (GFP), with low levels of transcription leakage from the *lac* promoter, was visually observed even in *E. coli* cells [13]. This suggests that TED may have a positive effect on translation rather than transcription. However, the mechanism underlying this effect remains unclear.

The insertion of a short sequence may enhance downstream gene expression in other bacteria; however, this is still unknown. Typically, the 20–50 a.a.-length short leader peptides were employed for a bicistronic design expression system [15–17]. In this system, a short peptide (the first cistron) is generally translated using the first SD sequence in the 5′UTR and a second SD site containing the first cistron initiates the translation of the target gene, which is usually called the second cistron, under the control of a single promoter [18,19]. In the case of several fungi, the insertion of a ~100 bp fragment containing the *cis*-element into a heterologous promoter substantially accelerates the expression of the downstream gene [20,21].

In order to elucidate the underlying mechanism of *mlcR25*-based TED, we analyzed whether the levels of mRNAs containing *mlcR25* and those without *mlcR25* are altered in vivo and investigated whether properties of the mRNA itself promote translation using in vitro translation experiments. Because TED has only been observed intracellularly, the components of the cell utilized for TED and whether the phenomenon is dependent on the genetic background of the cell are unclear. Hence, we aimed to reproduce TED in vitro using a reconstituted cell-free protein synthesis system (the PURE system) [22]. This system is ideal for quantitative analysis because proteins can be synthesized using chemically defined components, and both DNA and mRNA can be used as a template. Moreover, we tested the availability of TED in the purple photosynthetic α-proteobacterium *Rhodobacter capsulatus*, which has long been studied in the field of photosynthesis and is widely used as a host for the expression of recombinant proteins to understand the utility of the TED system in different bacteria. We hypothesize that TED leads to increased protein production through translation control of mRNA in various bacteria.

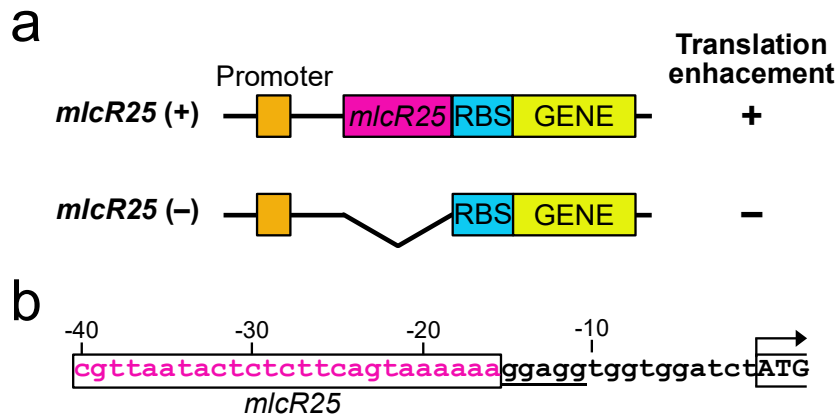

Figure 1. Representative gene structure for translation enhancement. (**a**) Translation enhancement is observed when *mlcR25* is inserted in the 5′-UTR [13]. (**b**) Nucleotide-sequence-containing *mlcR25* (shown in magenta). The Shine–Dalgarno (SD) sequence is underlined. The start codon is shown in uppercase.

2. Materials and Methods

2.1. Cell Culture

Escherichia coli K-12 derivatives DH5α and HST08 (Takara Bio, Shiga, Japan), which are widely used for molecular cloning, and XL1-Blue/pDPT51, which is used for transformation via conjugation in several bacteria, were cultured at 37 °C in lysogeny broth (LB) medium

in the presence of appropriate antibiotics [23]. Rifampicin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in ethanol at a concentration of 2 mg/mL, and a final concentration of 2 μg/mL was used for growing the cells. Trimethoprim and spectinomycin were used at a concentration of 50 μg/mL and 40 μg/mL, respectively.

Rhodobacter capsulatus SB1003 was cultured aerobically at 30 °C in PYS [24], a rich medium, or RCV minimum medium [25]. Gentamycin, rifampicin, and spectinomycin were used at a concentration of 1.5 μg/mL, 100 μg/mL, and 10 μg/mL, respectively.

2.2. Plasmids

The insertion of *mlcR25*-RBS-GFP (#154295; Addgene) or RBS-GFP into the pUC19 vector has been described previously [13]. *mlcR25*-RBS-GFP or RBS-GFP was inserted into the pET vector using restriction enzyme digestion with enzymes *Xba*I (Takara Bio, Kusatsu, Japan) and *Bam*HI (Takara Bio, Kusatsu, Japan), followed by ligation.

The *sqr* promoter and *lacZ* fusion plasmids were constructed using the pNM481 plasmid [26] with the Ω-interposon (Sm^r/Sp^r) gene [27] inserted upstream of the cloned fragment, which were transferred into *R. capsulatus* with the conjugative *E. coli* strain XL1-Blue/pDPT51, as described previously [28,29]. The insertion of *sqr* promoter into the pNM481 plasmid with the Ω-interposon gene has been described previously [30]. The insertion of *mlcR25* into the *sqr* promoter region was accomplished by amplification of the *sqr*-promoter-inserted pNM481 without the Ω-interposon gene (pNM481:p*sqr*) by PCR using KOD One with a *mlcR25* containing primers (5′-cgttaatactctcttcagtaaaaaaggagggacagatggc tcatatcgcc-3′) and (5′-tactgaagagagtattaacgaaaagccgaactggctgtcgggccgaagcc-3′) and circularization of the amplified DNA fragment by In-Fusion HD Cloning kit (Takara Bio, Kusatsu, Japan). The Ω-interposon (Sm^r/Sp^r) gene was then inserted into the plasmid at the *Sma*I site by ligation. Transformation of *E. coli* was performed chemically or by electroporation using the Gene Pulser Xcell system (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. In Vitro Transcription

Template plasmid DNA was prepared using the QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany). After linearization using *Eco*RI, DNA was purified using phenol-chloroform extraction and ethanol precipitation. The T7 RiboMAX Large-Scale RNA Production System (Promega, Madison, WI, USA) was used for in vitro transcription. The synthesized mRNAs were purified using phenol extraction, followed by isopropanol precipitation, and were stored at −80 °C until further use.

2.4. In Vitro Translation

PURE*flex* 1.0 (GeneFrontier Corporation, Tokyo, Japan) was used as a cell-free protein expression system according to the manufacturer's protocol. The composition of the system has been described previously [22,31]. A total of 10 μL of reaction mixture containing 5 μL of solution I, 0.5 μL of solution II, 1 μL of solution III, 2.5 μL of nuclease-free water, and 1 μL of DNA (180 ng) or mRNA (1 ng) was incubated at 37 °C for 6 h. Next, 10 μL of nuclease-free water and 20 μL of 3× Laemmli sample buffer were added to the reaction mixture to stop the reaction. The samples were stored at −20 °C until further use.

2.5. Western Blotting

The samples (3 μL) were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (0.2 μm pore size, Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked using 3% skim milk in PBS (137 mM NaCl, 2.7 mM KCl, 4 mM $Na_2HPO_4{\cdot}12H_2O$, and 0.7 mM KH_2PO_4; pH 7.4) for 1 h at 25 °C. After washing three times with PBS containing 0.05% Tween-20 (PBS-T), the membrane was incubated with rabbit anti-GFP antibody (MBL, Tokyo, Japan; Code No. 598) at 1:1000 dilution with Can Get Signal solution 1 (TOYOBO, Osaka, Japan) for 1.5 h at 25 °C. After washing with PBS-T three times, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Chicago, IL, USA) at 1:5000 dilution with Can Get Signal solution 2 (TOYOBO)

for 1 h at 25 °C. Blots were visualized using the enhanced chemiluminescence procedure with the Immobilon Western chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, USA) and imaged using an ImageQuant LAS 4000 (GE Healthcare, Chicago, IL, USA). A standard CBB solution or Quick-CBB PLUS solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used to stain the transferred proteins on the membrane. Band densitometry analysis was performed using the Fiji/ImageJ software [32]. The value for the GFP band was normalized to that of the control protein (~45 kDa) band observed in the same lane stained with CBB.

2.6. RNA Purification and Real-Time PCR

After culturing the cells in LB medium containing antibiotics, 7×10^8 cells in the logarithmic growth phase were collected by centrifugation and treated with lysozyme for 10 min at 37 °C. RNA was then purified using NucleoSpin RNA Plus (Takara Bio), according to the manufacturer's protocol. The RNA concentration was measured using NanoDrop (Thermo Fisher Scientific, Carlsbad, CA, USA). Then, 100 ng of RNA in the mixture was used for reverse transcription using the PrimeScript RT reagent kit (Takara Bio). Real-time PCR was performed with TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) in a 25 µL volume using a Thermal Cycler Dice Real Time System III (Takara Bio). Two cDNA dilutions (1:2 and 1:4) were used, and duplicates were set for each reaction. The extent of amplification was evaluated using the $2^{-\Delta\Delta Ct}$ method. The *cysG* was used as an internal control to normalize the cDNA input [33]. The following primers were used: *gfp* Forward: 5′-ggtgaaggtgaaggagatgc-3′ and Reverse: 5′-taggccagggtacaggtaac-3′, and *cysG* Forward: 5′-attgaacacggaatgccagg-3′ and Reverse: 5′-gtgagcgtaccgtcaatcac-3′.

2.7. β-Galactosidase Assay

Rhodobacter capsulatus cells containing the *sqr* promoter region and *lacZ* fusion plasmid were grown aerobically to the mid-log phase in RCV medium. For sulfide induction, a final measure of 0.6 mM of Na_2S was added and cells were grown further for 120 min. After the induction, 15 mL of cells were harvested, and β-galactosidase activity was determined essentially as described previously [28]. The results were obtained as the amount of o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per min per mg of protein.

2.8. Statistical Analysis

Statistical analyses were performed using GraphPad Prism9 (GraphPad Software, La Jolla, CA, USA). The unpaired *t*-test, one-way ANOVA, and post hoc Tukey's multiple comparisons test were performed as indicated. Normality was tested using the Shapiro–Wilk test or the Kolmogorov–Smirnov test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of mlcR25 on mRNA Longevity in E. coli

It has been demonstrated that the AU-rich sequence in the 5′-UTR stabilizes the mRNA [34]. No correlation between AT% and its enhancing effect has been found in TED [13]; however, since *mlcR25* comprises 72% of the AT-rich sequence (Figure 1b), the longevity of mRNA in cells harboring the plasmids with or without *mlcR25* under the control of the *lac* promoter (Figure 2a) was investigated [13]. Rifampicin, an inhibitor of the bacterial DNA-dependent RNA polymerase [35], was used to inhibit transcription. After rifampicin treatment, the total RNA was isolated from the cells at designated time points. The total amount of *gfp* mRNA without *mlcR25* (RBS-GFP) was found to have decreased over time, whereas that of the *gfp* mRNAs with *mlcR25* (*mlcR25*-RBS-GFP) was constant, suggesting that mRNAs containing *mlcR25* remained stable for a longer duration than those without (Figure 2b). Finally, the amount of *gfp* mRNA in the cells containing each plasmid was compared. Consistent with the increased longevity of *gfp* mRNA, the *gfp* mRNAs with *mlcR25* were found to be, on average, four times more abundant than those without *mlcR25* (Figure 2c). Thus, *mlcR25* had a positive impact on mRNA longevity. Since

a high mRNA content is assumed to increase the frequency of contact with the ribosome, the mRNA content is one possible cause of TED.

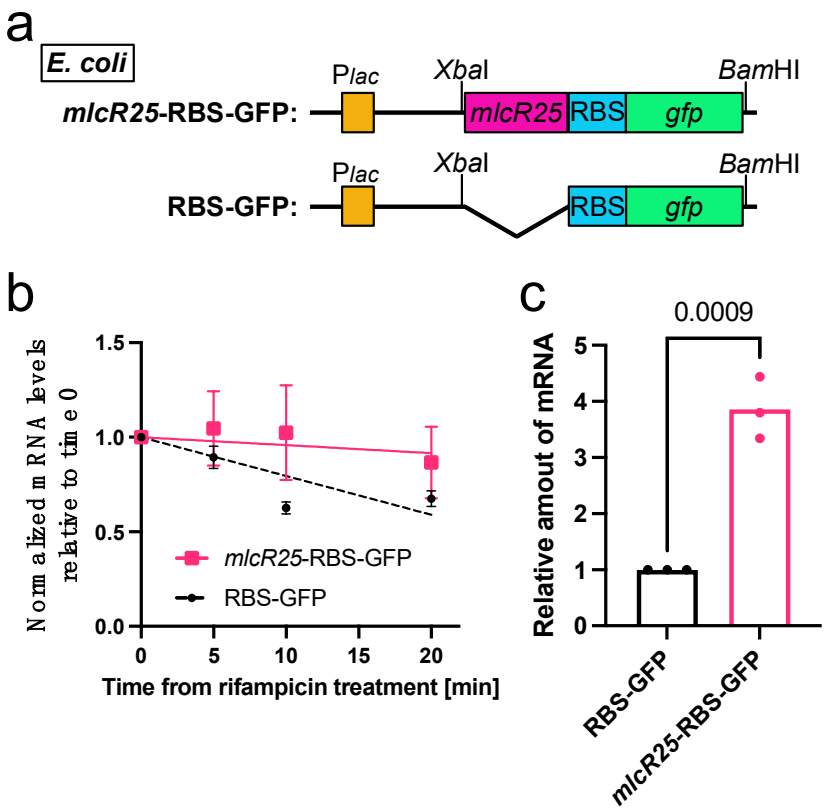

Figure 2. *mlcR25* in the 5′-UTR prolongs mRNA longevity in *E. coli.* (**a**) Gene structures with or without *mlcR25* under the control of *lac* promoter. (**b**) Relative amounts of indicated mRNAs over time after rifampicin treatment. The mean obtained from three independent experiments is shown. (**c**) Relative amounts of the indicated mRNAs obtained from the cells in the logarithmic phase. The mean obtained from three independent experiments is shown. *p*-values (unpaired *t*-test) are indicated.

3.2. mlcR25 Confers Enhanced Translational Efficiency on mRNA

An in vitro translation system was used to examine factors other than mRNA levels. A plasmid containing a ribosome-binding site (RBS) and inserted *mlcR25* to express GFP under the control of the T7 promoter to use in the PURE system [22,31] (Figure 3a) was constructed. The PURE system includes the T7 RNA polymerase and ribosomes to perform a series of mRNA and protein syntheses. First, DNA was used as a template to test whether TED works in the system. GFP (~27 kDa) synthesis from plasmids containing *mlcR25* was detected by Western blotting; however, that from plasmids without *mlcR25* was at a very low level (Figure 3b). The GFP band density was, on average, four times higher for *mlcR25*-RBS-GFP than for RBS-GFP (Figure 3c). These data demonstrate that TED can be reproduced in vitro, suggesting that it is driven by factors included in the PURE system.

Next, to examine whether mlcR25 promotes translation at the mRNA level, mRNA was used as a template in the PURE system. In the above experiment, owing to the addition of equal amounts of DNA to the reaction mixtures, equal amounts of GFP mRNA synthesized by T7 RNA polymerase were assumed to be present in the reaction mixture. The PURE system used does not contain mRNA-degrading enzymes, such as RNase E; thus, if *mlcR25* affects translation rather than transcription, even with the addition of equal amounts of mRNA in the system, an increase in the translation of *mlcR25*-RBS-GFP mRNA compared to that of RBS-GFP would be expected. An in vitro translation using mRNA instead of DNA was used to verify this hypothesis. The mRNA containing *mlcR25* showed, on average, three-times-higher protein synthesis than an equal amount of mRNA without

mlcR25 (Figure 3d,e). Hence, it was concluded that TED using *mlcR25* leads to increased protein expression through mRNA regulation at the translational level.

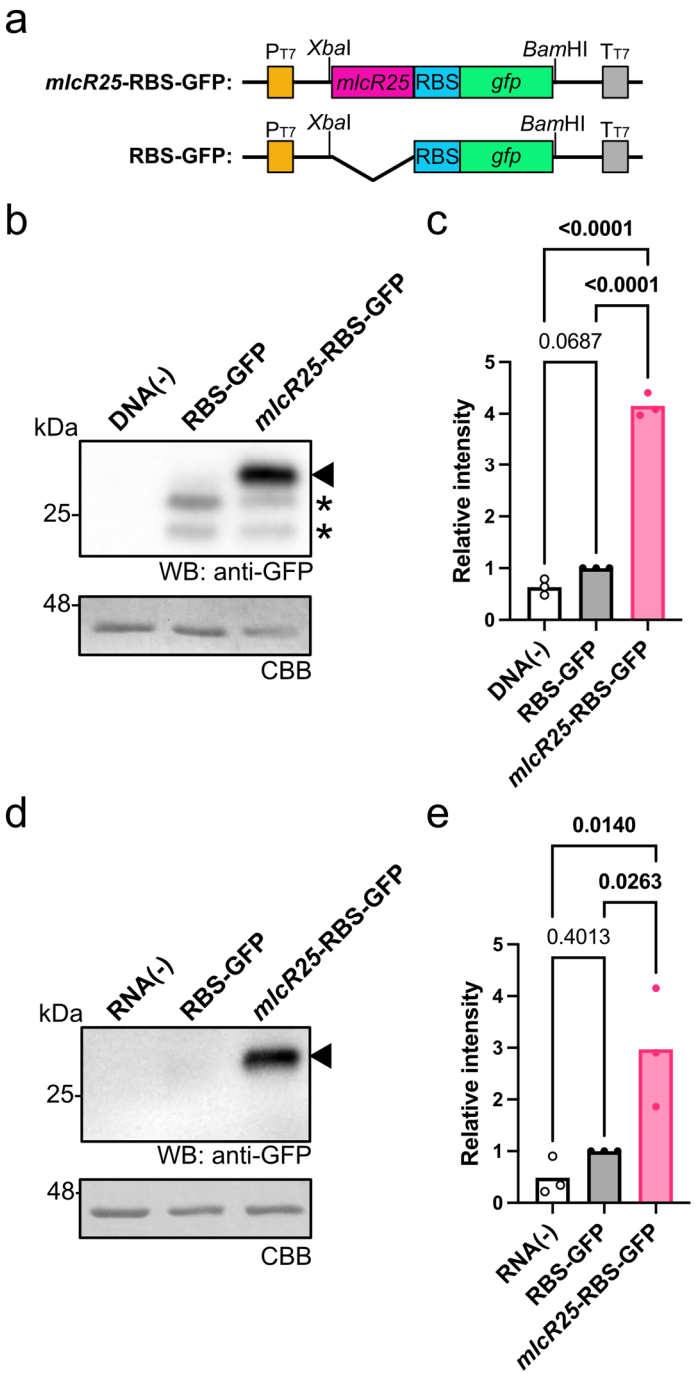

Figure 3. TED is observed in the in vitro translation system. (**a**) Gene structures with or without *mlcR25* under the control of the T7 promoter. (**b**) Western blot analysis of each reaction mixture using anti-GFP antibody. Plasmid DNA was added as the template. Arrowhead, GFP; asterisks, unknown proteins that were synthesized. (**c**) The relative band intensity of GFP normalized to bands of the control protein stained with CBB, related to (**b**). The mean obtained from three independent experiments is shown. (**d**) Western blot analysis of each reaction mixture using anti-GFP antibody. mRNA was added as the template. Arrowhead, GFP. (**e**) The relative band intensity of GFP normalized to bands of the control protein stained with CBB, related to (**d**). The mean obtained from three independent experiments is shown. *p*-values (one-way ANOVA followed by Tukey's multiple comparisons test) are indicated.

3.3. Application to a Photosynthetic Bacterium

To examine the effect of *mlcR25* in other bacteria, we used *R. capsulatus* with a *lacZ* reporter system driven by the *sqr* promoter (Figure 4a). In this construct, *mlcR25* was inserted upstream of *sqr* encoding sulfide-quinone reductase (SQR) and further fused with *lacZ*. The *sqr* promoter is repressed by sulfide-responsive transcription factor SqrR in the absence of sulfide [30]. Therefore, the *sqr* promoter driving *lacZ* expression was induced by treating the cells with sulfide, and the translation level of *lacZ* was subsequently measured as its enzymatic activity. Importantly, we found that the insertion of *mlcR25* upstream of the SD sequence clearly increased the activity of β-galactosidase by about sixfold (Figure 4c). This suggests that TED functions in other bacteria as well as in *E. coli*.

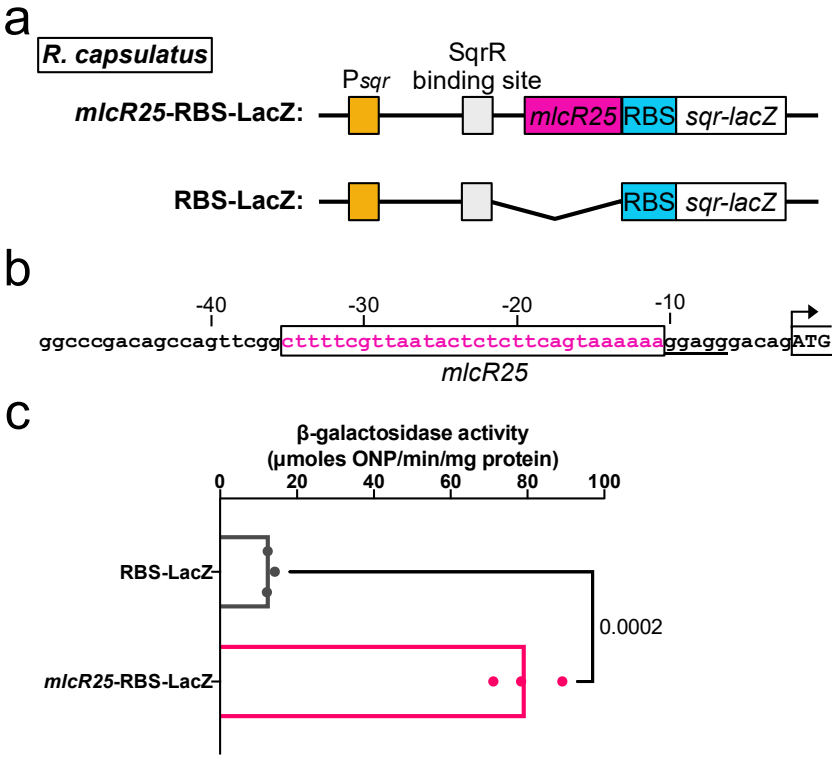

Figure 4. TED is applicable to the protein expression system of *R. capsulatus*. (**a**) Gene structures with or without *mlcR25* under the control of *sqr* promoter, which is placed at 1238 bp upstream from the *sqr* start codon. (**b**) Nucleotide sequence containing *mlcR25*, which is shown in magenta. The SD sequence is underlined. The start codon is shown in uppercase. (**c**) Measurement of β-galactosidase activity driven by the *sqr* promoter. The enzymatic activity was calculated as the amount of o-nitrophenol (ONP) hydrolyzed from ONPG by β-galactosidase. The mean obtained from three independent experiments is shown. *p*-values (unpaired *t*-test) are indicated.

4. Discussion

In this study, it was observed that *mlcR25* extended the lifespan of mRNA in *E. coli* cells. We demonstrated that the insertion of *mlcR25* upstream of RBS promotes translation in vitro, regardless of DNA or RNA being used as the template, similar to the effect observed in cells. Furthermore, the translation-enhancing effect of *mlcR25* was also observed in *R. capsulatus*, a bacterium from a different class.

For in vitro translation, we used the PURE system [22,31], which consists of highly purified proteins required for transcription, translation, aminoacylation, and energy regeneration as well as amino acids and NTPs and demonstrated that *mlcR25*-mediated TED is facilitated during the translation process in the system. The following components are included in this system: IF1/IF2/IF3 as initiation factors, EF-Tu/EF-Ts/EF-G as elongation factors, RF1/RF2/RF3 as release factors, ribosome recycling factor, 20 kinds of aminoacyl-tRNA synthetase, and methionyl-tRNA transformylase. The fact that the components

necessary for transcription–translation are known is an advantage because it eliminates unknown material in the cell. Hence, we deemed this system to be appropriate for the purpose of this study.

Cell-free systems such as the PURE system have been used to synthesize membrane proteins, which are generally difficult to obtain, and antibodies for mRNA/ribosome display [36–39]. The PURE system is expected to be a fundamental method for synthetic biology [40,41], and improvements in product solubility and synthetic cost are still being made [42,43]. We suggest that the method using *mlcR25* presented in this paper is useful because it can be immediately adapted to regulate the expression efficiency.

We used *R. capsulatus* as a model case for the TED-adapted non-*E. coli* protein expression system. This bacterium develops an intracytoplasmic membrane when growing photosynthetically in order to perform photosynthesis effectively by increasing the surface area of the membrane; therefore, *R. capsulatus* and phylogenetically closely related *R. sphaeroides* are utilized for the overexpression and purification of membrane proteins [44–46]. *Rhodobacter capsulatus* is also used for the functional expression of cofactor-dependent enzymes because it can produce a wider variety of metal-containing coenzymes as compared with *E. coli* [47]. Several vectors have also been constructed to optimize and control the expression of these various proteins [48]. Therefore, the availability of TED in this bacterium has significant bioengineering potential. Taxonomically, *E. coli* is a γ-proteobacteria, whereas *R. capsulatus* belongs to the class α-proteobacteria. The transcriptional mechanisms of RNAP/σ70 of *E. coli* and *R. capsulatus* are likely similar because they are structurally and functionally similar in part [49]. In translation, the SD sequences are conserved in the genes of *R. capsulatus* and *R. sphaeroides* [50,51] as in other bacteria [52]. Although there are differences between the ribosomes of *E. coli* and *R. capsulatus* [53,54], we assume that there are similarities in the translation mechanism, at least at the initiation stage. Thus, TED may be adaptable to the broad bacteria.

It remains unclear how the insertion of *mlcR25* in the 5′-UTR helps in the ribosome function. Komarova et al. (2005, [34]) reported that AU-rich sequences promote the binding of ribosomal protein S1. This protein is known to contribute to mRNA unfolding during translation initiation [55]. Furthermore, translation is suppressed when there is a stable secondary structure in the RBS [56]. Therefore, AU-rich sequences should be placed in this region to reduce the stability of mRNA and increase its acceptability by ribosomal protein S1. Interestingly, *mlcR25* has been predicted to form a secondary structure with neighboring sequences [13]. The relationship between *mlcR25* and ribosomal protein S1 should be investigated in the future.

The reason for the stability of mRNAs containing *mlcR25* for a long duration is unclear. One possibility is that they are stabilized owing to the formation of a stem-loop structure that is less susceptible to degradation, as observed in studies pertaining to *ompA* [57–59]. The deletion of a 104 bp sequence, including the region that forms the stem loom upstream of the SD of innate *ompA*, promotes mRNA degradation. Importantly, the insertion of synthetic sequences with 5′ self-complementarity to create a stem loop restores the stability. Thus, based on our prediction of the stem loop created by *mlcR25* [13], such secondary structures may contribute to the observed mRNA stabilization. Another possible mechanism for the longevity of the mRNAs containing *mlcR25* is the coupling of transcription and translation. In bacteria, transcription and translation occur simultaneously (i.e., the ribosome binds and translation begins before transcription is completed) [3,7,60]. One scenario is that the transcribed mlcR25-containing mRNA is immediately bound to the ribosome and translated, which may result in protection from RNase and increase its net mRNA content. mRNAs that are translationally inefficient are actively degraded [61]. On the contrary, highly translated mRNAs are less likely to be degraded [2,9,62]. Similarly, a previous study on *ompA* has also indicated that ribosome binding at the 5′-UTR induced mRNA stability [58]. Considering these findings, an increased stability and level of mRNAs with *mlcR25* supports the fact that translation is promoted by *mlcR25*.

5. Conclusions

In conclusion, TED with *mlcR25* promotes translation by acting as a cis-acting factor in *E. coli*, *R. capsulatus*, and the PURE system. The fact that translation was promoted by mRNA in the PURE system in this study suggests that *mlcR25* promotes the interaction of mRNAs containing this sequence with ribosomes. Additionally, the amount of mRNA containing *mlcR25* was also increased, which also increases the frequency of contact between mRNA and ribosome, and thus may help promote the translation of downstream genes. In addition to the improvement of the gene structure shown in this study, further improvement of the transcription apparatus and ribosome is also possible at the genomic level, and tuning both elements should be emphasized in the future.

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Conflicts of Interest: The authors declare no conflict of interest.

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