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Real time determination of bacterial *in vivo* ribosome translation elongation speed based on LacZα complementation system

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ABSTRACT

Bacterial growth significantly depends on protein synthesis catalyzed by ribosome. Ribosome translation elongation speed is a key factor determining the bacterial protein synthesis rate. However, existing methods for determining translation elongation speed have limited applications. Here we developed a simple and convenient method for measuring bacterial translation elongation speed based on LacZα complementation system. It enables the measurement of *in vivo* translation elongation speed of different individual genes. Tests related to ribosome translation elongation speed under various growth perturbations including different nutrient conditions, low temperature, a low-speed ribosome mutant, and fusidic acid treatment, were performed to quantitatively validate this method. Using this approach, we further found that nutrient starvation caused a remarkable slow-down of ribosome translation of *Escherichia coli* (*E. coli*). We also studied the dynamic change of translation elongation speed during the process of nutrient up-shift. This method will boost the quantitative understanding of bacterial ribosome translation capacity and growth.

INTRODUCTION

Quantitative understanding on cell growth under various growth conditions is a very challenging task in the recently emerging field of systems biology (1). Protein accounts for over half of the bacterial cellular biomass and its synthesis consumes about two-thirds of the total cellular energy cost, and thus, protein synthesis catalyzed by ribosome plays a central role on bacterial growth (2,3). *In vivo* protein synthesis rate comprises two parameters: ribosome translation elongation speed (ribosome activity) and ribosome content (ribosome number) (2). The ribosome content can be obtained conveniently by measuring the bacterial RNA/protein ratio (given 86% of RNA is rRNA and rRNA is co-regulated with its affiliated proteins) (4,5). For measuring ribosome translation elongation speed, currently there are two approaches, pulse-chase radioactive labeling (6) and β-galactosidase (LacZ) induction assay (7,8). The former approach can, in principle, measure the translation elongation speed of each individual gene. However, it requires dual radioactive labeling and further 2-D gel separation of individual proteins, which is non-trivial for obtaining high-quality data. The latter approach measures the translation time of newly synthesized LacZ after adding Isopropyl β-D-Thiogalactoside (IPTG), which is much more simple and convenient. However, it only measures the translation elongation speed of *lacZ* gene, and doesn't account for the time cost of the transcription and translation initiation steps (Supplementary Figure S1).

Here we report a new method for measuring the translation elongation speed of bacteria based on the well known LacZα complementation system (9) (Supplementary Figure S2). The basic principle of this method is similar to that of the LacZ induction assay. However, it can rapidly and conveniently obtain the translation elongation speed of each individual gene, and can also distinguish the time cost of the transcription and translation initiation steps from that of the elongation step.

MATERIALS AND METHODS

Strains

All the strains used in this study are derivatives of three strains: wild type *Escherichia coli* K-12 MG1655 strain, streptomycin pseudo-dependent (SmP) CH184 strain (10), and B/r AS19 strain (11). CH184 harbors a hyper-accurate ribosome mutant with a much slower translation elongation speed than wild type strain. AS19 strain is permeable to fusidic acid.

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Construction of pKUT15 series vectors

The pKUT15 vector is derived from a very low-copy plasmid pZS24*MCS (SC101* replicon). The *lacZα* fragment (N-terminal residues 1–60 of LacZ) was PCR amplified and inserted into the KpnI/MluI site of pZS24*MCS plasmid, yielding pZS24*-LacZα. The long upstream primer of *lacZα* fragment also contains a sequence coding for $(GGGGS)_2$ linker so that the linker coding sequence was in frame fused with LacZα fragment. The native *lac* promoter (−200 bp to −6 bp relative to the *lacZ* transcription starting point) was amplified from the genome of MG1655 strain and cloned into the HindIII/KpnI sites of pZS24*-LacZα to replace the native Plac/ara-1 promoter, this yields pZS24*-Plac-LacZα. The coding sequence of *lacZω* fragment was amplified from the genome of *E. coli* Top10 strain and cloned into the KpnI/BamHI sites of pZA31-luc, thus being under the control of *Ptet* promoter, yielding pZA31-*lacZω.* The fragment containing *Ptet-lacZω*-T_1 (T_1 terminator) was further PCR amplified from pZA31-*lacZω* and cloned into the SalI/HindIII sites of pZS24*-Plac-LacZα. Finally using point mutation, a pair of XhoI/NotI sites were introduced between the transcription site of native *lac* promoter and $(GGGGS)_2$ linker coding sequence to facilitate the fusion between target genes and *lacZα*. This finally yields pKUT15 vector. For the measurement of translation elongation speed, *fusA*, *tufA* genes were PCR amplified with MG1655 genome as template, while the *bla* was amplified with plasmid pBR322 as template. The fragments of these three genes were finally inserted into the XhoI/NotI sites of pKUT15 to be in frame fused with *lacZα*.

Construction of LacZ-deficient strain

To measure translation elongation speed using our LacZα complementation system, we need to transform the pKUT15 series vectors to a LacZ-deficient strain. Strain JW0332 (*ΔcynX782::kan, ΔlacZ4787 (::rrnB-3)*) was obtained from the coli genetic stock center (CGSC, Yale university). This strain carries a *ΔcynX782::kan* mutation, which is adjacent to the *lac* operon. Deletion of the *cynX* gene causes no phenotype defect. Moreover, this strain is LacZ deficient due to carrying the *ΔlacZ4787 (::rrnB-3)* allele which has the start codon of *lac* operon removed. Allele of *ΔcynX782::kan, ΔlacZ4787 (::rrnB-3)* was transferred to all the three strains, MG1655, CH184 and AS19 through P1 transduction to obtain their LacZ-deficient counterparts. The *kanR* gene was then flipped out using pCP20 plasmid. pKUT15 series vectors were then transformed to the LacZ-deficient strains for measurement of translation elongation speed.

Medium

The growth medium used in this study is MOPS buffered minimal medium described at Neidhardt *et al*. (12). For minimal medium, carbons were used as below: 0.2% (w/v) glucose (for starvation experiment 0.05% and 0.4% were used), 0.2% (w/v) fructose. In addition, rich defined medium (RDM) contains 0.2% (w/v) glucose, various amino acids, nucleotides, micronutrients, and vitamins as described in Neidhardt *et al* . Glucose + cAA medium contains 0.2% (w/v) glucose and 0.2% (w/v) casamino acids. For all the above media, 10 mM NH_4Cl (for starvation assay, 4 mM and 15 mM were used) was supplied as nitrogen source.

Cell growth

Cell growth was always performed in an air bath shaker (25°C or 37°C) shaking at 240 rpm. Cell growth always contained three steps, seed culture in LB broth, pre-culture and experimental culture in MOPS minimal medium. For seed culture, cells from a fresh solid LB plate was inoculated into LB liquid medium and cultured at 37°C. After several hours, cells were centrifuged and washed with fresh MOPS minimal medium, and then inoculated into the same MOPS minimal medium for overnight growth (at 25°C or 37°C) as pre-culture. In the next day, pre-culture was diluted into identical fresh MOPS minimal medium starting from OD_{600} around 0.01–0.02 as experimental culture. About 6–10 OD_{600} data points within the range of ∼0.05 and ∼0.5 were taken for calculating the growth rate. At OD_{600} around 0.4 or 0.5, cell samples were taken for translation elongation speed measurement. For all the cells harboring pKUT15 series vectors, 30 μg/mL kanamycin was always supplied into the culture.

Measurement of translation elongation speed

The basic principle is similar with the classical LacZ induction assay as described in Andersson *et al.* (13). *E. coli* cells harboring pKUT15 series vectors were grown to OD_{600} around 0.4 to 0.5 (for ammonia starvation study, the OD_{600} finally reached 0.7), 5 mM IPTG was added to the culture to induce the expression the *lacZα* fused gene in the pKUT15 vector. At a time interval of 5–15 s (this depends on the translation elongation speed, can be 5, 10 or 15 s), around 15–20 cell samples (1 ml each) were immediately pipetted into pre-chilled eppendorf tubes containing 10 μl chloramphenicol (34 mg/ml) for blocking further translation and then quickly frozen in liquid nitrogen and stored at –80°C before measurement. Then before LacZ activity measurement, cell samples were incubated at 37°C water bath for 1 hour to allow the new synthesized LacZα fragment to totally complement with LacZω fragment. The LacZ activity of cell samples was measured using a sensitive fluorescence substrate 4-methylumbelliferyl-D-galactopyranoside (MUG) (14,15). 400 μL cell sample was added to 100 μl five X Z-buffer and pre-warmed at 37°C water bath for 10 min. 100 μl MUG stock (4 mg/ml) was then added and the reaction mixtures were incubated for 0.5–2 h. The reaction was then stopped by 300 μl 1 M Na_2CO_3. The fluorescence intensity was measured with a micro-plate reader (365 nm excitation filter, 450 nm emission filter).

Calibration of the time cost of the initiation step (T_{init})

To calibrate the time cost of the initiation steps (including the IPTG penetration, LacI de-repression, RNA polymerase transcription initiation, and ribosome translation

initiation), a control pKUT15 vector with only LacZα fragment (no other genes fused) was used to obtain the induction kinetic of the 70 aa LacZα fragment (containing 10 aa linker) for all the growth conditions studied in this work. The synthesis time of the LacZα fragment (T_α) was estimated using the straight-line fit of the induction kinetics data. For the same conditions, the synthesis time of Protein A-LacZα fused protein (T_A) was also obtained. The time cost of the initiation step (T_{init}) equals to $T_\alpha - 70/ [L_A/(T_A - T_\alpha)]$ (L_A means the length of A protein).

RESULTS

Method establishment

A low-copy vector called pKUT15 was constructed for this method (Figure 1A). This vector contains a *lacZα* fragment (coding for the N-terminal residues 1–60 of LacZ) driven by the native *lac* promoter. A pair of XhoI and NotI sites was placed downstream of the *lac* promoter where the target gene can be inserted and be fused in frame with the *lacZα* fragment by a $(GGGGS)_2$ linker to guarantee its freedom (16,17). Moreover, the vector also contains a *lacZω* fragment driven by a constitutive *Ptet* promoter. To test our system, we chose three target genes, *fusA*, *tufA* and *bla*, whose translation elongation speeds under some conditions were available (6,18). The *lacZ* gene in the wild type cell was further inactivated before using this vector.

Steady-state growth of *E. coli* MG1655 strain harboring the pKUT15 vector was almost unaffected compared to that of the control strain under all the testing conditions (Supplementary Figure S3), suggesting that the very low-copy pKUT15 vector did not cause obvious physiological burden for *E. coli*. When IPTG was added to exponentially growing cultures of the *E. coli* strain harboring pKUT15, synthesis of the LacZα fused protein was induced. The newly synthesized fused protein complemented with the existing excess amount of LacZ*ω* fragment (Supplementary Figure S4) to yield LacZ activity (Supplementary Figure S2). Thus, we obtained the induction kinetics of the fused proteins (Figure 1B, Supplementary Figure S5A and C). Moreover, the exact translation time of the first fused proteins was also obtained by using the 'Schleif plot' as in the case of the classical LacZ induction assay (7) (Figure 1C, Supplementary Figure S1, and Figure S5B and D). The time cost of initiation steps (T_{init}) including the IPTG penetration, LacI de-repression, RNA polymerase transcription initiation and ribosome translation initiation, which is ignored in classical LacZ induction assay (Supplementary Figure S1), is addressed by repeating the induction kinetic of the empty LacZα fragment without target proteins fused (Figure 1D and Supplementary Figure S6). For example, as shown in Figure 1D, the synthesis time of LacZα (T_α) in glucose + cAA medium at 37°C was 14 s. At the same conditions, the synthesis time of FusA-LacZα protein (T_{fusA}) was 58.7 s. Therefore, T_{init} equals to T_α-70/ [704/ (T_{fusA}-T_α)] (The length of FusA protein is 704 aa), so we can obtain that T_{init} equals to 9.6 s. Using this strategy, we found that the initiation steps always took around 10 s in all our test conditions (Supplementary Figure S6). Therefore, the translation elongation speed was obtained easily by using the length of the LacZα fused protein to divide its translation elongation time (caption of Figure 1, Supplementary Figure S6, and Table S1). The error bars of different rounds of experiments were within 10% (Supplementary Figure S7).

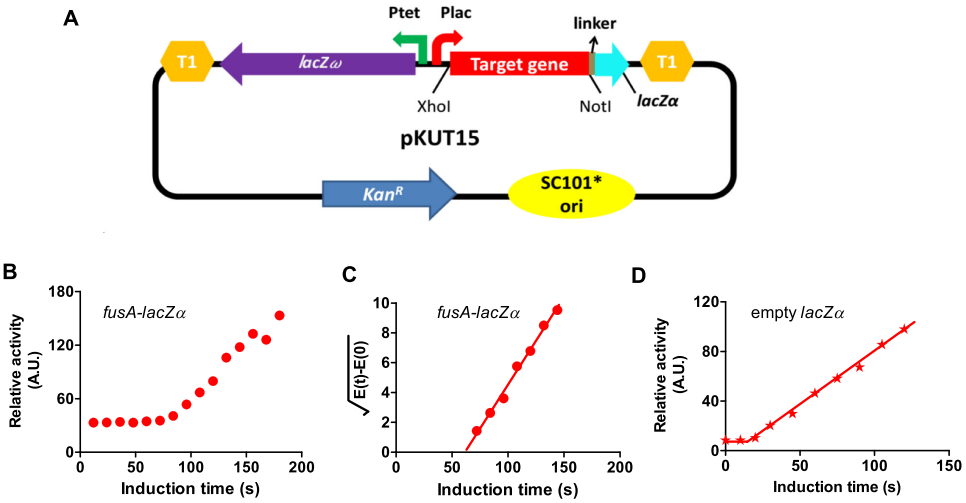


Figure 1. (**A**) pKUT15 vector designed for measuring translation elongation speed. (**B**) Induction kinetics of FusA-LacZα protein. Cells were grown in glucose + cAA medium at 37°C. (**C**) Schleif plot of FusA-LacZα induction kinetics in panel (B). The square root of the newly synthesized enzyme was plotted against the induction time. The X intercept, which is 58.7 s in this condition, denotes the translation time of the FusA-LacZα protein (T_{fusA}). (**D**) Calibration of the time cost of initiation steps (T_{init}) by measuring the induction kinetics of the empty LacZα fragment (with no target gene fused). MG1655 cells harboring the empty LacZα were grown in glucose + cAA medium (same as panel B and panel C) and the induction kinetic of the empty LacZα was also measured. The T_{init} was found to be always around 10 s (Supplementary Figure S6). Therefore, the translation elongation time of the FusA-LacZα protein (panel C) is 48.7 s. The length of FusA-LacZα is 774 aa so the translation elongation speed is 774/48.7 = 15.9 aa/s.

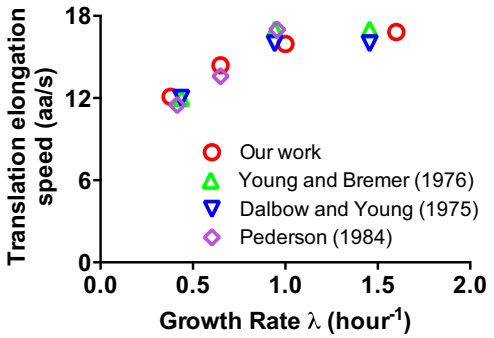


Figure 2. Translation elongation speed of MG1655 upon nutrient limitation at 37°C (Red open circles). The studied protein was FusA-LacZα. Data points were plotted together with previous results conducted at similar growth range.

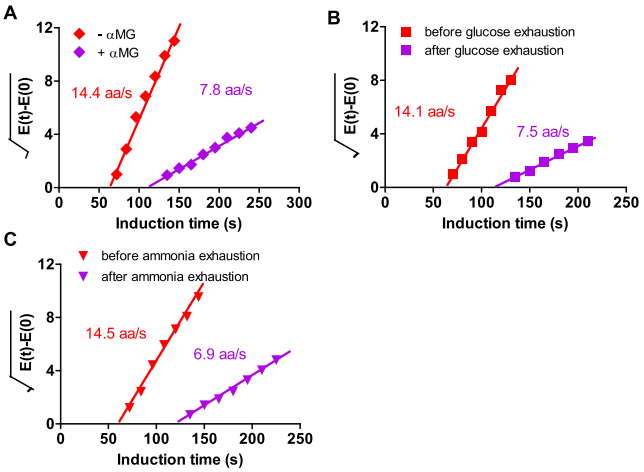


Figure 3. Translation elongation speed of MG1655 strain under starvation. The data shown are Schleif plots of FusA-LacZα induction kinetics. (**A**) Translation elongation speed of MG1655 strain in 0.2% glucose medium before (red) and after (purple) the addition of 2% α-methylglucoside (αMG) (Supplementary Figure S11A). (**B**) Translation elongation speed of MG1655 strain in glucose medium before (red) and after (purple) glucose exhaustion. Cells were grown with 0.05% glucose and 15 mM NH_4Cl so growth arrest occurred at OD_{600} around 0.5 due to glucose exhaustion (Supplementary Figure S11B). (**C**) Translation elongation speed of MG1655 strain in glucose ammonia medium before (red) and after (purple) ammonia exhaustion. Cells were grown with 0.4% glucose and 4 mM NH_4Cl so growth arrest happened at OD_{600} around 0.7 due to ammonia exhaustion (Supplementary Figure S11C).

Method tests in various conditions

Several rounds of tests were performed to validate this method. We first studied the translation elongation speed of MG1655 strain upon nutrient limitation at 37°C. We took FusA-LacZα (774 aa) protein as the example. Cells were grown in MOPS minimal medium supplemented with different nutrient sources to achieve different growth rates. The result clearly showed that the translation elongation speed is growth-rate dependent upon nutrient limitation. Translation elongation speed saturated at around 16–17 aa/s at fast growth (GR > 1/h), but decreased at slower growth (Figure 2). This is consistent with the results of several previous sporadic studies at similar growth range using other methods (6,8,19) (Figure 2).

When *E. coli* grows at low temperatures, both its growth rate and translation elongation speed substantially drop in proportion to each other (20). The translation elongation speeds of all the three genes, *fusA-lacZα*, *tufA-lacZα*, and *bla-lacZα* were measured at 25°C using our method. The growth rates at 25°C were always only one third of those at 37°C. Strikingly, translation elongation speeds of all the three proteins were around four to six aa/s, which were also only about one third of those at 37°C (Table 1, Supplementary Figure S8, and Table S1). This is in good agreement with the results of previous studies on the slow-down of translation elongation at low temperatures (6). Moreover, similar as previous studies (6,18), *bla* is always translated at a lower speed than *fusA* and *tufA*, which may be due to its higher content of rare codons (6).

To further validate our system, we measured the translation elongation speed of a translation mutant strain CH184. CH184 is a streptomycin pseudo-dependent strain (SmP) (10,21). It harbors a hyper-accurate mutant ribosome that translates much more slowly than normal ribosome. Translation elongation speeds were around 4–5 aa/s by using our method (Table 2). Moreover, the addition of 100 μg/ml streptomycin (Str), which reduces the proofreading cost of the mutant ribosome, stimulated both the growth rate and translation elongation speed by two-fold (Table 2, Supplementary Figure S9). This quantitatively reproduced existing data of the translation elongation speed of the SmP strain (Table 2) (10). This is also consistent with the value of LacZ protein at the same growth condition after initiation time calibration (Supplementary Figure S9D).

Fusidic acid (FA), a protein synthesis inhibitor, which prevents the turnover of elongation factor G (EF-G) from the ribosome, was also found to cause substantial slow-down of translation (11). This was again captured by our method. Using a B/r derived AS19 strain (11), which is permeable to fusidic acid, our method showed that 1 μg/ml of fusidic acid (sublethal dose), which reduces the growth rate by three-fold, caused a two-fold decrease in the translation elongation speed (Table 2, Supplementary Figure S10).

Translation elongation speed upon nutrient starvation and nutrient up-shift

All the above tests confirmed that our approach could effectively obtain the *in vivo* translation elongation speed. We then used this approach to study translation elongation speed under starvation. Upon nutrient starvation, the overall protein synthesis rate substantially decreases (22). However, information of the translation elongation speed remained unknown. We studied both glucose (carbon) starvation and ammonia (nitrogen) starvation. Glucose starvation was triggered by adding either an excess amount of α-methylglucoside (αMG, a non-metabolizable glucose analogue) (23) or by direct glucose exhaustion of the medium (Supplementary Figure S11A and B). Ammonia starvation was triggered by direct ammonia exhaustion of the medium (Supplementary Figure S11C). Strikingly, the translation elongation speed of all three fused genes was reduced by about two-fold in all the three cases (Figure 3, Supplementary Table S2), suggesting that the decrease of translation

Table 1. Effect of temperature on translation elongation speed

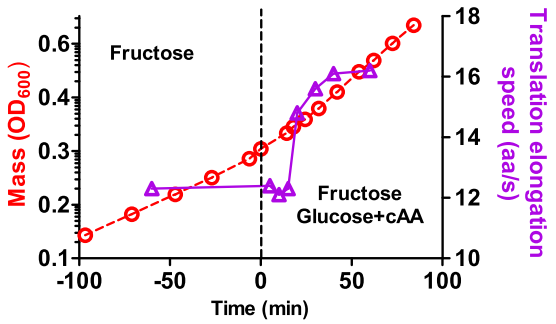
medium	Translation elongation speed[c] (aa/s)					
	fusA-lacZα		*tufA-lacZα*		*bla-lacZα*	
	37°C	25°C	37°C	25°C	37°C	25°C
Glucose + cAA[a]	15.9	6.0	16.2	5.8	13.6	4.2
Glucose[b]	14.4	5.4	13.9	5.0	11.9	4.1

[a]Growth rates in glucose+cAA medium at 37°C and 25°C were 1 h^{-1} and 0.34 h^{-1}, respectively.
[b]Growth rates in glucose medium at 37°C and 25°C were 0.65 h^{-1} and 0.2 h^{-1}, respectively.
[c]Measurements were repeated for three times and deviations were within 10%.

Table 2. Translation elongation speed of CH184 strain upon Streptomycin (Str) treatment and AS19 strain upon Fusidic acid (FA) treatment

Strain	Translation elongation speed[c] (aa/s)					
	fusA-lacZα		*tufA-lacZα*		*bla-lacZα*	
CH184 (SmP)[a]	+ Str	- Str	+ Str	- Str	+ Str	- Str
	10.1	5.4	10.5	5.0	8.4	4.5
AS19[b]	+FA	-FA	+FA	-FA	+FA	-FA
	8.9	16.4	8.1	16.1	6.1	13.6

[a]Grow rate of CH184 (harboring each of the three fused proteins) in glucose+cAA medium with and without 100 μg/ml Str were 0.86 h^{-1} and 0.44 h^{-1}, respectively. The translation elongation speeds of the SmP strain were 5 aa/s and 11 aa/s for growth without and with 100 μg/ml Str respectively in Ruusala et al[15], which quantitatively agrees with our results.
[b]Growth rates of AS19 (harboring each of the three fused proteins) in glucose medium with and without 1 μg/ml FA were 0.3 h^{-1} and 0.97 h^{-1}, respectively.
[c]Measurements were repeated for three times and deviations were within 10%.



Time	Translation elongation speed (aa/s)
Before Shift	12.3
5 min after shift	12.4
10 min after shift	12.1
15 min after shift	12.3
20 min after shift	14.8
30 min after shift	15.6
40 min after shift	16.1
60 min after shift	16.2

Figure 4. Dynamic change of translation elongation speed of MG1655 strain during the nutrient up-shift process. MG1655 cells harboring FusA-LacZα protein was first grown exponentially on minimal medium with 0.2% fructose to OD_{600}~0.25. At $t = 0$ (dashed vertical line), 0.2% glucose plus 0.2% casamino acids are supplemented for starting the up-shift process. Optical density, OD_{600} (red open circles) and translation elongation speed (purple triangles) are plotted versus time.

elongation speed accounts for a significant fraction of the dramatic reduced overall protein synthesis rate upon starvation. It is interesting to study whether the substantial increased ppGpp pool upon starvation plays any role on this process (22–24). However, how those factors quantitatively cause the 2-fold drop in translation elongation speed demands more work.

In the natural living condition, bacteria seldom undergoes steady-state growth, instead may frequently undergo changing environment. Media shift study has long been used for studying the regulation and adaption strategy of bacteria in changing environments (25,26). However, information regarding the translation elongation speed during the media shift process is still unclear. Here we investigate the translation elongation speed during nutrient upshift process. MG1655 strain harboring FusA-LacZα protein was first grown in minimal medium with 0.2% fructose as the sole carbon source. During exponential growth (OD_{600}: 0.25), 0.2% glucose + 0.2% casamino acid was directly added to the medium to start the nutrient up-shift process. The time course of the OD_{600} of the cell culture was measured throughout the whole process (Red circle, Figure 4); faster growth occurred rapidly after the shift ($t = 0$, dashed vertical line). Eight of the same nutrient-shift experiments are performed in parallel so that the translation elongation speed of the FusA-LacZα protein was measured at eight different time points (Before up-shift and 5, 10, 15, 20, 30, 40 and 60 min after shift). Translation elongation speed didn't change within 15 min after the media shift but then rapidly increased and reached almost saturation at around 30 min after media shift. In the future, it is interesting to study the molecular mechanism underlying the pattern of translation elongation speed change. It is also conceivable to extensively perform similar analysis in other kind of media shift experiments such as nutrient downshift and also

shift to some stress condition (such as low or high temperature, pH, oxidative stress and so on).

DISCUSSION

In summary, we have developed a rapid and convenient method for measuring bacterial *in vivo* translation elongation speed based on the well-known LacZα complementation system. Compared with the pulse-chase radioactive labeling, it is simple and convenient to be applied by a routine lab. Compared with the classical LacZ induction assay, it not only enables the translation elongation speed measurement of other individual genes, but also can distinguish the time cost of initiation steps that is ignored in classical LacZ induction assay. Using the method, we showed that translation elongation speed substantially dropped upon starvation, contributing a substantial fraction to the dramatically reduced overall protein synthesis upon starvation. We have also studied the dynamic change of the translation elongation speed during a nutrient up-shift process.

This method will boost our understanding of *in vivo* translation capacity regulation. Moreover, owing to the high robustness of the LacZ reporter system and LacZα-complementation in many other bacteria, it is conceivable to apply this method to other bacteria such as some important pathogens as a more general approach in the future. To date, our understanding on the translation capacity and growth of many other bacteria is still limited. This method, together with ribosome content (which can be obtained by measuring RNA/protein ratio (5)), will help us understand the differences on translation capacity and growth physiology of various bacteria.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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