The Role of the C-terminal Tail of the Ribosomal Protein S13 in Protein Synthesis

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Contents

Abbreviations

Amp – ampicillin

ATP – adenosine triphosphate

bp – base pair

Cm – chloramphenicol

CTD – c-terminal domain

DC – decodng center

DMSO – Dimethyl sulfoxide

DNA – deoxyribonucleic acid

DTE – dithioerythriol

EDTA - Ethylenediaminetetraacetic acid

EF – elongation factor

GTP – guanosine triphosphate

H38 – helix 38

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IF – initiation factor

Kan – kanamycine

MK – myokinase

mRNA – messenger RNA

nt - nucleotide

OD – optical density

PCR – polymerase chain reaction

PEG – polyethylene glycol

PEP – phosphoenolpyruvic acid

PK – pyruvate kinase

PM – polymix

PMSF – phenylmethanesulfonylfluoride

RNA – ribonucleic acid

Rpm – revolutions per minute

rRNA – ribosomal RNA

RS – aminoacyl-tRNA synthetase

SD sequence – Shine-Dalgarno sequece

TBE - Tris/Borate/EDTA

TFA – trifluoroacetic acid

Tris - tris(hydroxymethyl)aminomethane

tRNA – transfer RNA

wt – wild type

Abstract

Ribosomal protein S13 has length of CTD tail that differs from species to species; when comparing the S13 CTD tail of *Escherichia coli* and *Thermus thermophilus*, *T. thermophilus* has a longer CTD tail which is seen to be closer to, and interact more with the a-site tRNA and p-site tRNA in ribosome structures. In order to study the role of the CTD tail of ribosomal protein S13, we created four different strains of *E. coli* derived from the strains MG1655 and JE28. Based on the alignment of protein S13 sequences from *E. coli* and *T. thermophilus*, we have truncated or extended the CTD tail of the S13 protein in *E. coli*. We measured the growth rate of the 8 different strains as well as mean tripeptide formation times *in vitro* using ribosomes purified from our JE28 derived strains. The CTD tail was not essential for bacterial growth; however modification of this tail certainly leads to defects in generation time and translocation. While the *E. coli* CIK28c strain (7 aa extension; similar to CTD tail in *T. thermophilus*) and the CIK28a strain (4 aa shortening) shows the largest growth defects, the CIK28c strain has greater time defects in translocation as compared to the CIK28a strain. Further experiments are needed to determine the detailed function of the CTD tail in ribosomal protein S13.

1. Introduction

1.1 Ribosomal protein S13

1.1.1 Structure of the Prokaryotic Ribosome

The ribosome is the largest macromolecular machine, and it synthesizes all proteins in every cell based on the genetic information encoded in mRNA. The 70S ribosome in prokaryotes is composed of two subunits; called the 30S and 50S subunits. The 30S subunit consists of the 16S rRNA (~1540 nucleotides) and 21 proteins whereas the 50S consists of the 23S rRNA (~2900 nt), the 5S rRNA (~120nt) and 31 proteins (1).

The functional sites of the ribosome are the decoding center (DC) and the peptidyl transferase center (PTC) (2). The PTC which is located in the 50S subunits catalyzes peptide bond formation between the two amino acids bound to the A-site and P-site tRNA. The decoding center of the ribosome is mainly made up of the rRNA of the small subunit. This forms the channel which allows mRNA to go through and match the corresponding tRNA codons; thus improving the fidelity of translation (3, 4).

1.1.2 Ribosomal protein S13 in *Escherichia coli* and *Thermus thermophilus*

Ribosomal protein S13 is located in the head part of the 30S ribosome subunit and interacts with several other proteins and rRNAs. In *E. coli* protein S13 which is 118 amino acids long has 5 helices and a few positively charged amino acids in the CTD. This protein locates in the head of the 30S ribosomal subunit near the subunit interface and interacts with P-site bound tRNA through ribosomal protein L5 at the N-terminal domain as well as directly at the CTD (Figure 1). It also interacts with the A-site tRNA via H38 of the 23S rRNA.
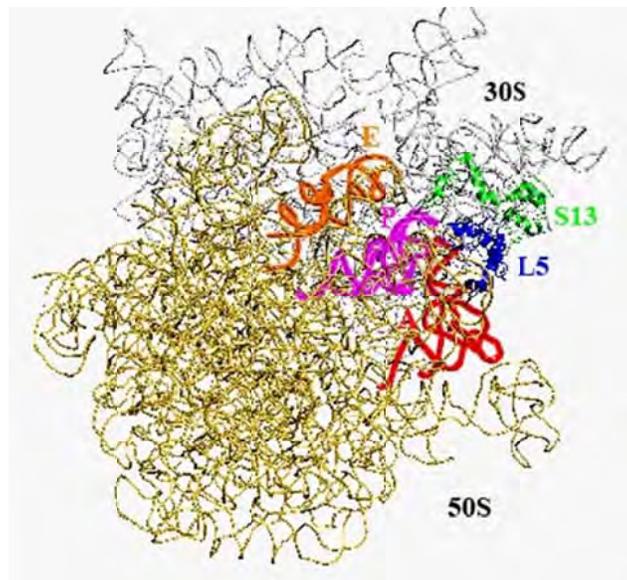

Figure 1. Location of protein S13 in ribosome (PDB: 3E1C, 3E1D)

Cukras and Green (2005) have indicated that absence of this protein in *Escherichia coli* ribosomes induces considerable defects in growth rate, subunit association as well as translocation during protein synthesis. It is also suggested that protein S13 is linked to maintenance of the pretranslocational state (6).

In *Thermus thermophilus*, ribosomal protein S13 which is 126 amino acids the CTD tail is seen close to the decoding center (DC), but it is still unknown what this tail does in translation. The C terminal tail of protein S13 is located between the A-site tRNA and the P-site tRNA (Figure 2) and therefore likely affects translocation. However, the CTD of S13 in *E. coli* is shorter and not extended near the DC compared to the one in *T. thermophilus*.
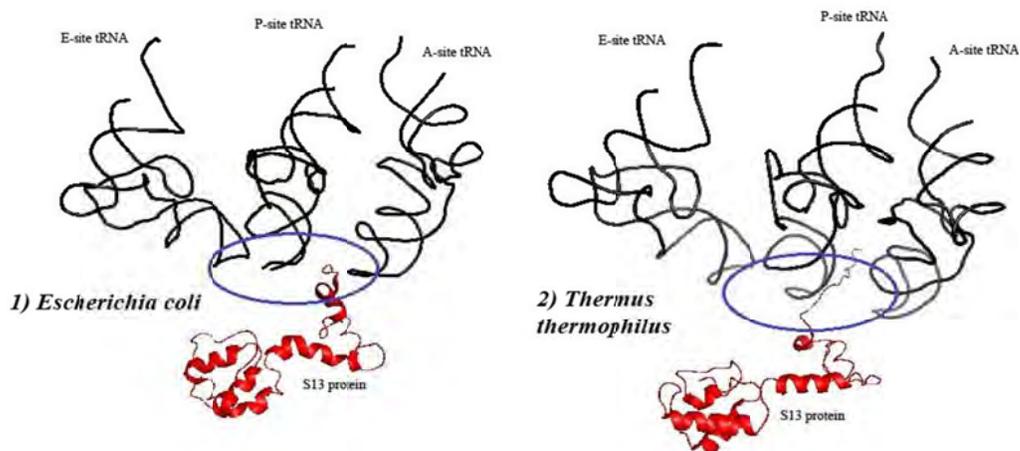
Figure 2. The location and structure of the protein S13 in *E.coli* and *T. thermophilus* (3E1C, 4K0L) ribosome in relation to three tRNAs bound to A-, P- and E-site. The decoding center is circled in this figure.

1.2 Chromosomal engineering in *Escherichia coli*

1.2.1 λ-red recombineering

λ-red recombineering has been developed based on the mechanism of integration and excision of bacteriophage λ. λ-red recombination technology utilized the recombination function and enzymes of phage λ (17, 18).

λ-red recombineering is a highly effective method for constructing chromosomally engineered *Escherichia coli* strains. This technique is far more convenient than restriction enzyme systems since it can manipulate any sequence in the genome or a plasmid while restriction enzyme based methods require specific restriction sites. λ-red recombineering only requires a short (40-60 nt long) homologous sequence matching the target sequence in the genome and linear DNA cassettes can easily be designed and produced by PCR. The important recombineering enzymes are Gam, Beta and Exo; Gam prevents RecBCD nuclease from degrading double-strand linear DNA fragments while Exo and Beta produce 3' overhang and bind, bring it to the target respectively (7, 8).

1.2.2 Structure of pSIM5 plasmid

The pSIM5 plasmid was used for λ-red recombineering. It encodes recombineering enzymes including Exo, Beta and Gam, as well as the chloramphenicol resistance gene – chloramphenicol acetyl-transferase (cat) (Figure 3). The CI857 protein is a temperature sensitive repressor which enables production of recombineering enzymes only at a specific temperature (9).
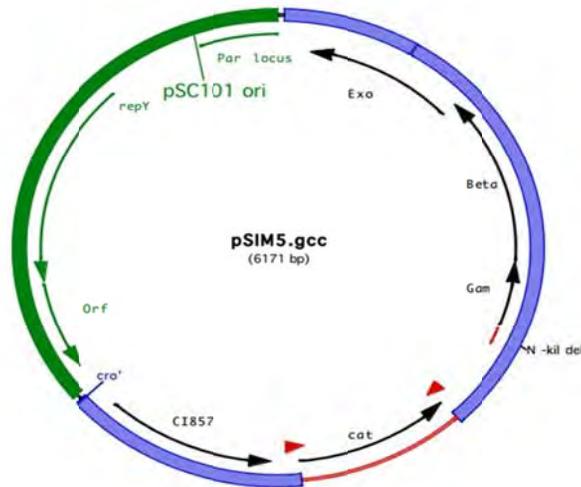

Figure 3. Structure of pSim5 plasmid
(http://redrecombineering.ncifcrf.gov/Plasmids_files/pSIM5.pdf)

1.3 *Escherichia coli* JE28

Escherichia coli MG1655 is a wild type strain whilst *Escherichia coli* JE28 is a new strain that was developed in the Sanyal lab at the Department of Cell and Molecular Biology, Uppsala University in 2008. This strain (derived from the parental strain HME6) encodes a hexa-histidine affinity tag at the 3'-end of the *rplL* gene as well as a kanamycin resistance gene; thus enabling single step bacterial ribosome purification which is convenient for research as it enables purification of ribosomes in a shorter time and more effectively compared to conventional purification methods (11).

1.4 In vitro characterization of ribosomes

In order to characterize the function of ribosomes and other factors affecting translation, in vitro experiments have been used. To provide an optimal ion environment for the ribosome stability and translation polymix buffer was developed (13, appendix c), and the purification methods of every factor including initial factors, elongation factors, release factors and aminoacyl-tRNA synthetases was established.

Radioactive amino acids (normally radioactive labeled fMet) and chromatography are used to screen the process of reaction. Short peptide formation or whole protein synthesis can be used

depending on which you are interested in. In this project we used MLL tripeptide formation assay to determine translocation of different strains.

1.5 Aim of the project

The aim of this study is to determine the possible function of the CTD tail of ribosomal protein S13 by chromosomally engineering the S13 gene (rpsM) in *Escherichia coli*. As can be seen in the Figure 4, the CTD tail of *T. thermophilus* S13 has some extra amino acids compared to the *E. coli* one. The following constructs were designed (Figure 4.a, a-d). For easy selection of the recombineered strains an ampicillin resistance cassette was added after the target sequence. The λ-red recombineering strategy for chromosomal engineering of rpsM is also shown in Figure 4.
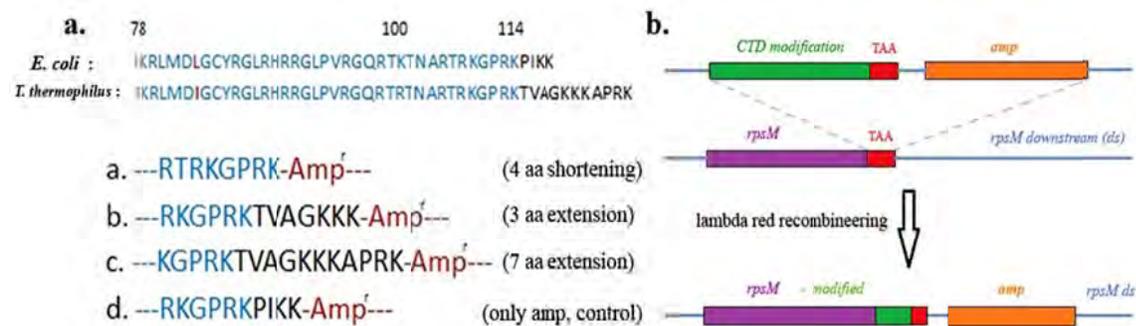

Figure 4. **a**. Aligned sequences of S13 CTD from *E. coli* and *T. thermophiles*
a) is a shortening of protein S13 while b) and c) are a short and a longer extension respectively.
d) is the control including only the Amp resistance gene.
b. Strategy for CTD tail modification by lambda red recombineering

2. Materials and Methods

2.1 *Eschericha coli* strains and plasmids

For this project *Escherichia coli* MG1655 with pSIM5 plasmid which is chloramphenicol resistant and *Escherichia coli* JE28 strain which is kanamycin resistant were used. These strains were grown on plates with antibiotics (50 µg/ml of chloramphenicol for *E. coli* MG1655 and 50 µg/ml of kanamycin for *E. coli* JE28) from glycerol stock.

Plasmid pND707 was used as template for PCR in order to extract the ampicillin resistance gene bla.

2.2 Engineering protein S13 in *Escherichia coli*

2.2.1 Amplification of DNA cassettes

The ampicillin resistance cassette was amplified from the plasmid pND707 using the primers listed in appendix a (1-5). The primers have 30 ~ 40 nt homologous to the *rpsM* gene, followed by the modified S13 CTD sequence, a stop codon, an *E. coli* SD sequence for translation of amp resistance gene, and short sequence complementary to the amp resistance gene in pND707. The reverse primer was the same; each forward primer had a different S13 CTD sequence before its stop codon. The primers were purchased from Invitrogen. The primers were centrifuged at 14000 rpm for 10 min and dissolved in EB buffer (10 mM Tris-Cl, pH 8.5) to 100 mM stock, which was in turn diluted to 10 mM for the PCR reaction.

A standard PCR protocol was used (commercial Taq DNA polymerase master mix (Thermo Scientific) was used and the manufacturer's instructions were followed); the program used was initial denaturation at 94 ° C for 5 min followed by 30 cycles of 94 ° C for 30 sec, 58 ° C for 30 sec and 72 ° C for 1 min, and then final extension at 72 ° C for 5 min.

An agarose gel was prepared to separate the amplified DNA cassettes. The amplified DNA cassettes were electrophoresed on 1% agarose gel (50 ml 1×TBE buffer, 0.2 µg/ml Ethidium Bromide, and 0.5 g agarose (Sigma)), and then purified using a commercial gel purification kit (Qiagen). The concentration was measured and the cassettes were stored at -20 ° C for λ- red recombineering.

2.2.2 Transformation of pSIM5 to *E. coli* JE28 strain

Plasmid pSIM5 was extracted from *E. coli* MG1655+pSIM5 using a QIAGEN midi kit.

E. coli JE28 strain was grown overnight before transformation in 10 ml LB + 50 µg/ml kanamycin. The 1 ml of overnight culture was then diluted 100-fold with pre-warmed 100 ml LB and grown for around 1 h 40 min until OD_{600} was around 0.5. After that it was immediately cooled by shaking on ice for 2 min and transferred to a falcon tube to be centrifuged at 4000 rpm for 15 min at 4 ° C. The pellet was resuspended in 5 ml (1/20th of the start volume) of ice cold TSB buffer (1 × LB with 10 % PEG4000, 5 % DMSO, 10 mM of $MgCl_2$ and 10 mM of $MgSO_4$) and incubated on ice for 10 min.

20 ng of pSIM5 plasmid was mixed with KCM buffer (100 mM KCl, 30 mM $CaCl_2$ and 50 mM $MgCl_2$) and cooled (Table1). Then 100 µl of competent JE28 cells were added, the mixture was incubated on ice for 20 minutes and then at room temperature for another 10 minutes. Next, 1 ml of LB media was added into the mix, and the cells were grown at 37 ° C for 1 hour. The cells were spun again at 14000 rpm at 4 ° C, resuspended in a small volume (200 µl) of ice cold water and spread on a 50 µg/ml LA plate.

2.2.3 Electroporation

Escherichia coli strains MG1655 and JE28, containing plasmid pSIM5 were prepared for electroporation by the following procedure; 0.5 ml of overnight (O/N) culture (30 ° C, LB with 50 µg/ml chloramphenicol) was added in 50 ml of pre warmed LB with 50 µg/ml chloramphenicol and grown until an approximate OD_{600} value of 0.5 (around 2 h 30 min). In order to express recombineering enzyme the cells were then incubated at 42 ° C, 110 rpm for 15 min immediately followed by 10 min incubation on ice. After that the cells were transferred to 50 ml tubes, collected by centrifugation (4000 rpm, 4 ° C, 10 min). Next, the supernatant was removed, and the pellet was rinsed with 15 ml of ice cold sterile 10 % glycerol and centrifuged again (4000 rpm, 4 ° C, 5 min). The cells were rinsed with ice cold 10 % glycerol again and collected. Finally, the cells were resuspended in 300 µl of ice cold 10 % glycerol for electroporation.

300-500 ng of synthesized new DNA cassettes and 50 µl electro-potent *E. coli* strains (MG1655, JE28) in 10 % glycerol solution were put in pre-chilled electroporation cuvettes and incubated on ice for 5 min. They were then electroporated (2.5 kV, 25 µF, 200 Ohms), immediately mixed with 1 ml LB media and transferred to eppendorf tubes for recovering (30 ° C, 180 rpm shaking overnight). Next day the cells were collected by centrifugation (14000 rpm, 2 min), resuspended in 150 µl LB, and plated on 50 µg/ml ampicillin plates for selection.

2.2.4 Selection & confirmation

To confirm the identity of the inserts in the engineered strains colony PCR (2 × Taq DNA polymerase master mix 10 µl, F primer 2 µl, R primer 2 µl, water 6 µl. The program was initial denaturation at 94 ° C for 5 min, 30 cycles of 94 ° C for 30 sec, 58 ° C for 30 sec and 72 ° C 2 min 30 sec, and final extension at 72 ° C for 5 min. This PCR confirmation was conducted using S13 wF primer as forward primer (5'- CTGTGCGTTT CCATTTGAGT ATCCTG -3') and S13 w Rev primer as reverse primer (5'- GCCGTCAGAG ACTTGTTTTC TTACAC -3'). These primers flank the whole rpsM gene so that the PCR amplification will yield a sequence of 450 bp in case the cassette has not inserted and 1500 bp if the cassette has been inserted correctly. PCR products were electrophoresed on 1 % Agarose gel (0.2 µg/ml Ethidium Bromide), extracted from the gel using QIAGEN gel purification kit, and sent for sequencing.

After the sequencing result confirmed that the correct insertions were present, the strains were grown at 37 ° C on the 50 µg/ml ampicillin LA plates 5 ~ 6 times to remove the pSIM5 plasmid. If the

strains could no longer grow on cm plates, they were grown in 10 ml of LB (with 50 µg/ml ampicillin) at 37 ° C and stored in 20 % glycerol at -80 ° C.

2.3 Growth Measurement

Each strain was grown overnight in 10 ml LB media with antibiotics (CIK28a – d: 50 µg/ml amp + 50 µg/ml kan, CIK1655a – d: 50 µg/ml amp, JE28: 50 µg/ml kan, MG1655: no antibiotics) from the plate. The overnight culture was then diluted 1000 times to reach an OD_{600} below 0.01. Next, 200 µl of diluted culture as well as fresh LB media for background was put in wells on the plate.

The TECAN/BioScreen recorded the OD_{600} value with an interval of 5 minutes maintaining temperature as well as shaking the plate between measurements. Background absorbance was subtracted from each data point and OD_{600} values of 0.03 ~ 0.08 were selected for analysis.

2.4 Ribosome purification

2.4.1 Ribosome purification using His-tag affinity Chromatography

Escherichia coli strains were grown overnight in 20 ml LB + 50 µg/ml antibiotics and then diluted 100 times to pre-warmed LB culture (with antibiotics). The cells were harvested by centrifugation (4000 rpm, 25 min, 4 ° C) and directly used or stored at -80 ° C.

The pelleted cells were resuspended by 30 ml of lysis buffer (Tris-Cl 20 mM pH 7.5, $MgCl_2$ 10 mM, NaCl 200 mM, Glycerol 5 %, DNAse 1 µg/ml, and PMSF protease inhibitor 100 uM) and lysed using French Press. After cell lysis, cell debris was removed by centrifugation 3 times (18000 rpm, 4 ° C, 20 min each).

The lysis solution was then applied to a column (Ni-NTA agarose, QIAGEN cat. no. 30230) at the speed of 1 ml/min using an ÄKTA prime followed by wash buffer (Tris-Cl 20 mM pH 7.5, $MgCl_2$ 10 mM, NaCl 200 mM, Glycerol 5 %, Imidazole 5 mM) to eliminate unspecifically bound molecules. The his-tagged ribosome was finally eluted by high imidazole concentration elution buffer (Tris-Cl 20 mM pH 7.5, $MgCl_2$ 10 mM, NaCl 200 mM, Glycerol 5 %, Imidazole 150 mM). The ribosome solution was dialysed against PM buffer and concentrated in PM buffer using an ultracentrifuge (35000 rpm, 4 ° C, 20 hrs) (12).

2.4.2 In vitro activity assay of his tagged ribosome

A Met-Leu dipeptide formation assay was used to measure the amount of active ribosomes in our samples. An equal amount of Ribosome mix (1 mM GTP, 1 mM ATP, 10 mM PEP, 1.2 µM mRNA, 0.05 mg/ml PK (sigma), 0.002 mg/ml MK, 1 µM 70S ribosome, 2 µM IF1, 1 µM IF3, 2 µM IF2 and 2 µM 3H-fMet in PM buffer) and Factor mix (1 mM GTP, 1 mM ATP, 10 mM PEP, 0.05 mg/ml PK, 0.002 mg/ml MK, 200 µM Leucine, 10 µM tRNA-Leu, 0.5 µM LeuRS, 5 µM EF-Ts, 10 µM EF-Tu in PM buffer) were pre-incubated at 37 ° C for 10 min, mixed together and incubated at 37 ˚ C for 5 sec to form dipeptide. The reaction was quenched by addition of an equal volume of 50 % formic acid. The

quenched solution was then centrifuged for 15 min at 14000 rpm and the supernatant was discarded. The pellet was resuspended in 165 µl of 0.5 M KOH solution by vortex to hydrolyse the diesther bond between the peptide and the tRNA, followed by precipitation by addition of 13.7 µl of 100 % formic acid. The mixture was spun at 14000 rpm for 15 min, acid was added again and the mixture span a second time, and finally 160 µl of the supernatant was loaded in the HPLC tube.

The peptides were separated using RP-HPLC with a C18 column and a mobile phase consisting of 42 % Methanol, 58% H_2O and 0.1 % TFA.

2.5 Ribosome Characterization

A Met-Leu-Leu tripeptide formation assay was used to characterize the elongation kinetics of the different ribosomes. Equal amounts of Ribosome mix (1 mM GTP, 1 mM ATP, 10 mM PEP, 1.2 µM mRNA, 0.05 mg/ml PK (sigma), 0.002 mg/ml MK, 1 µM 70S ribosome, 1 µM IF1, 1 µM IF3, 1 µM IF2 and 1 µM 3H-fMet in PM buffer) and Elongation factor mix (1 mM GTP, 1 mM ATP, 10 mM PEP, 0.05 mg/ml PK, 0.002 mg/ml MK, 200 µM Leucine, 6 µM tRNA-Leu, 0.5 µM LeuRS, 1 µM EF-Ts, 6 µM EF-Tu and 40 µM EF-G in PM buffer) were pre-incubated at 37 ˚C for 15 min and mixed together to synthesize tripeptide. It was then quenched with 50 % formic acid at different time points using a quench flow machine. The sample was subjected to the same process as described in 2.4.2 and loaded on HPLC.

The peptides are separated from each other on the HPLC and the fractions of fMet, fMet-Leu, fMet-Leu-Leu were recorded. Non-linear curve fitting was used to extract the reaction mean-times.

3. Results

3.1 Confirmation of DNA cassettes insertion

3.1.1 Purified DNA cassettes

The DNA cassettes were successfully amplified from the pND707 template (Figure 5) and purified (Table 1), these were in turn used for λ-red recombineering.
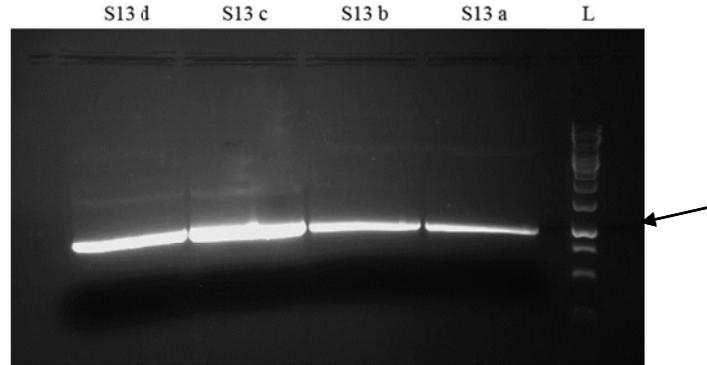

Figure 5. Four DNA cassettes for λ-red recombineering on the gel

The size of DNA cassettes was around 1 kb which is similar to the ampicillin resistance gene – bla. Each cassette has a different CTD modification sequence.

Table 1. Purified PCR products

	230nm Abs	260nm Abs	280nm Abs	260/280	260/230	Concentration (ng/µl)
S13 a	14.46	3.78	2.03	1.86	0.26	189.2
S13 b	14.77	4.98	2.69	1.85	0.34	249.1
S13 c	13.71	5.09	2.76	1.85	0.37	254.7
S13 d	13.06	5.89	3.16	1.86	0.45	269.4

3.1.2 PCR and sequencing confirmation of the recombineered strains

The engineered *Escherichia coli* strains were selected on 50 µg/ml ampicillin LB plates and subjected to repeated ampicillin selections to ensure that only resistant strains would survive. Then the correct insertion of DNA cassettes in the strains was confirmed by colony PCR (Figure 6). Engineered strains from JE28 produce exactly the same bands (around 1.5 kb: rpsM & amp resistance gene) while host strains produce a shorter band (~ 0.5 kb: rpsM only). (The colony PCR result figure of MG1655 and strains from MG1655 was not shown since they are basically the same.)

The sequencing results also show that all DNA cassettes were inserted correctly in the correct place (data not shown).
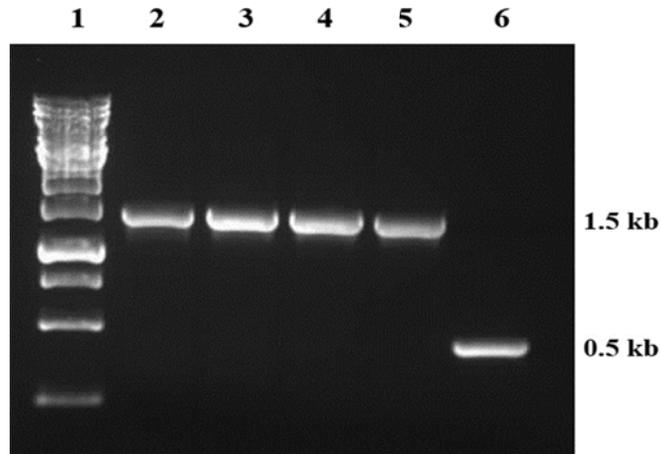
Figure 6. Colony PCR confirmation of engineered strains
1: DNA ladder, 2: CIK28a, 3: CIK28b, 4: CIK28c, 5: CIK28d, 6: JE28 wt

3.2 Modified protein S13 tail affects growth rate

As can be seen in Figure 7, *Escherichia coli* CIK28c and CIK28a strains show the largest growth defect when comparing to the control strain (CIK28d). The generation time of CIK28c is around 1.3 times longer than for CIK28d this might be because it has a long tail located between a-site tRNA and p-site tRNA and this tail might inhibit translocation of tRNAs along with the mRNA. CIK28a and CIK28c strains also grow slower at different temperatures (30 ˚C, 42 ˚C). (Data not shown)
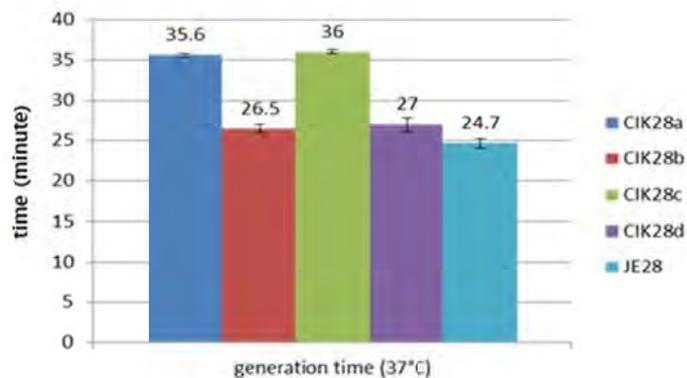

Figure 7. Generation time of CIK28 strains and JE28 wt

3.3 Modifications in protein S13 tail slows down translocation

3.3.1 Purification of ribosomes using his-tag affinity is more productive than conventional methods

The ribosome concentration was around 15 µM after being dissolved in a small volume of PM buffer. Around 10,000 pmol of ribosomes were purified from ~ 2 grams of cells, which is a higher yield than for conventional methods such as ultra-centrifugation. However, they had a clear brown color during dialysis, because the Ni from the resin might be eluted together with ribosome, interact with DTE in PM buffer and add the color to the ribosome solution (according to the manufacturer's instruction). This might be the cause for the low activity of his tag purified ribosomes (20 ~ 30 %).

3.3.2 Translocation of mRNA and tRNA in ribosomes from CIK28c strain is slower than wild type

From the MLL tripeptide assay, the mean reaction times of the dipeptide and tripeptide formation were estimated by non-linear fitting of the equations to the data (Figure 8). From those times the translocation time was then calculated.
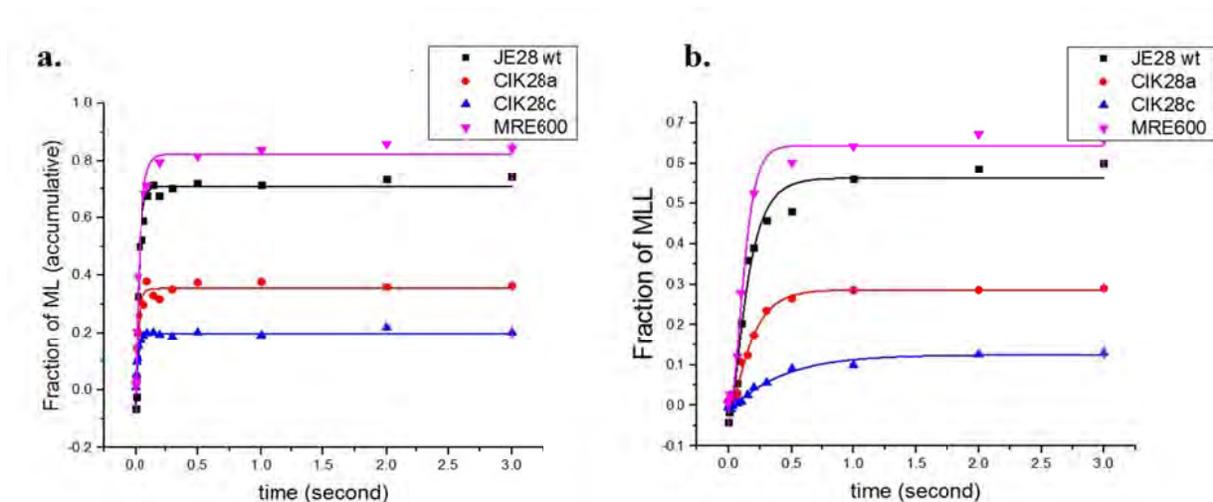

Figure 8. Plotted data to the fitting curve
a. dipeptide formation data, b. tripeptide formation data

As can be seen in Table 2, while there is little difference in dipeptide formation time between the ribosomes, the time for tripeptide formation differs depending on the ribosomes of our strains. In *E. coli* CIK28c strain ribosomes with T. thermophilus's long CTD tail, translocation takes around 4 times longer than for the wild type; while JE28 wt strain ribosomes translocate in 115 ms, in the ribosome of the CIK28c strain translocation takes around 400 ms. In addition, there is also a slight defect in translocation for the CIK28a ribosome of 4 aa truncation of protein S13 CTD tail.

Table 2. Ribosome characterization of each *E. coli* strains

strain	Dipeptide formation time ($\tau_1 \tau_3$, ms)	Tripeptide formation time (τ, ms)	Translocation time (τ_2, ms)
MRE600	34 ± 3.9	146 ± 18.5	78 ± 19.3
JE28	28 ± 2.3	171 ± 18	115 ± 18.7
CIK28 a (4 aa shortening)	25 ± 3.8	205 ± 19	155 ± 19.7
CIK28 c (full extension)	22 ± 2.5	450 ± 57	406 ± 57

4. Discussion

Protein translation is one of the most important processes for all living organisms; the ribosome is an indispensible macromolecular complex in every cell. Based on my results, modification of the CTD tail of ribosomal protein S13 affects the growth of *Escherichia coli*; especially the *E. coli* CIK28c (full extension) strain which has the long tail from *T. thermophiles*, shows the largest growth defect of all strains. One of the reasons might be the long translocation time measured for ribosomes from the CIK28c strain. It takes around 4 times longer than the wild type, which affects translation and in turn the whole growth time. The long CTD tail of CIK28c strain which is located between p-site tRNA and a-site tRNA certainly delays translocation; however how they affect it is not certain yet. Moreover, the reason why *Thermus thermophilus* has this long CTD tail on protein S13 which slows down translocation in *E. coli* needs to be determined by further experiments.

In addition, when considering the translocation time of the ribosomes from the CIK28a strain (four aa shortening), even though there is not as much difference as in the case of CIK28c strain, the growth defect is almost the same as for the CIK28c strain. This means that there might be other steps that affect the growth rate such as ribosome recycling, accuracy in tRNA selection and peptide release.

In this project the same strains were derived from *E. coli* MG1655 and the generation time was measured. Even though those data are preliminary they show a different pattern of growth defects; for example whereas the CIK28b strain (3 aa extension from JE28 wt) showed little defect, the CIK1655b strain (3 aa extension from MG1655) shows the highest growth defect among all 4 strains. The main difference between the two home strains of MG1655 and JE28 is the his-tag at CTD of L12 protein in JE28 strain (11), and we need to confirm how much this tetra his-tagged protein interacts with other components in the ribosome.

An ampicillin resistance gene was used as a selection marker in order to check the correct insertion during the engineering. In the JE28 derived strains the control strain (CIK28d: insertion of amp only) showed some difference in generation time compared to the wt, while the CIK1665d strain grows more slowly than MG1655. Since the S13 protein is the very first protein in the α operon (~ 3 kb, rpsM, rpsK, rpsD, rpoA, rplQ) (17), it seems that the insertion of amp resistance gene downregulates expression of other genes; thus, the insertion of amp (~ 1 kb long) could certainly affect the growth of the engineered strains.

In the ribosome purification process used, we encountered considerable problems with ribosome activity using Ni-NTA resin (QIAGEN, cat. no. 30230). This resin had not been used before in the Sanyal lab; Josefine Ederth et al used a Hi trap column to purify his-tagged ribosomes which was high quality and already packed. In the resin used before Ni might have been weakly bound to the agarose resin so that it was eluted together with the ribosome, which showed strong brown color during dialysis in PM buffer, it is possible that the reducing agent DTE reacted with Ni and changed the color. Several methods were used to remove the color like centrifugation at 14000 rpm after dialysis or by addition of high concentration histidine (1 mM) to compete with the ribosome from Ni binding. However, these were not effective in increase the portion of active ribosome to levels comparable to MG1655 wt.

Long extension of the S13 CTD tail slows down single translocation by ~4 times and shortening of the S13 CTD tail also shows some defect in translocation (~ 30 %). Whether the long extension stabilizes the pre-translocation state remains to be seen. Further experiments like accuracy, tRNA drop-off and ribosome recycling need to be carried out to determine the exact function of the protein S13 CTD tail.

Acknowledgement

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Appendix

Appendix a. List of primers used in the experiment

Primer name	Length (nt)	Sequence (5'-3')
S13 aF	75	ACCAAGACCAACGCACGTACCCGTAAGGGTCCGCGCAAATAATGAAAAAGGAGAGTATGAGTATTCAACATTTCC
S13 bF	91	GACCAACGCACGTACCCGTAAGGGTCCGCGCAAAACGGTGGCGGGCAAGAAGAAGTAATGAAAAAGGAGAGTATGAGTATTCAACATTTCC
S13 cF	99	AACGCACGTACCCGTAAGGGTCCGCGCAAAACGGTGGCGGGCAAGAAGAAGGCCCCGAGGAAGTAATGAAAAAGGAGAGTATGAGTATTCAACATTTCC
S13 dF	73	CACGTACCCGTAAGGGTCCGCGCAAACCGATCAAGAAATAATGAAAAAGGAGAGTATGAGTATTCAACATTTCC
S13 Rev	62	CGTGCACGAATTGGTGCCTTTGCCATTATTCAATCACCCCGATTACCAATGCTTAATCAGTG
S13 check	27	GTGGCCCGTATAGCAGGCATTAACATT
S13 middle	20	GTTGCCAAATTTGTCGTTGA
Amp int Rev	20	CATTGCTACAGGCATCGTGG
S13 w F	26	CTGTGCGTTTCCATTTGAGTATCCTG
S13 w R	26	GCCGTCAGAGACTTGTTTTCTTACAC

*All primers were purchased from Invitrogen.

Appendix b. engineered strains and description

Name	Host strain	Description
CIK1655a	MG1655	4 aa shortening
CIK1655b	MG1655	3 aa short extension
CIK1655c	MG1655	7 aa full extension
CIK1655d	MG1655	Control
CIK28a	JE28	4 aa shortening
CIK28b	JE28	3 aa short extension
CIK28c	JE28	7 aa full extension
CIK28d	JE28	Control

Appendix c. Preparation/Composition of buffers

Composition of 1× polymix buffer

components	KCl	NH$_4$Cl	Mg(OAc)$_2$	CaCl$_2$	putrescine	spermidine	DTE	HEPES
Concentration (mM)	95	5	5	0.5	8	10	1	10

Preparation of 10 × TBE buffer (1 L)

component	
Tris base	108 g
Boric acid	55 g
0.5M EDTA (pH 8.0)	40 ml
H$_2$O	Up to 1 L

Appendix d. Non-linear Fitting Curve Functions

One step: $Y = A_0 (1 - e^{-kt})$

Three step: $Y = \dfrac{k1k2k3A0}{k2-k1} \left(\dfrac{-1}{k1(k3-k1)} e^{-k1t} + \dfrac{1}{k2(k3-k2)} e^{-k2t} + \dfrac{1}{k3} \left(\dfrac{1}{k3-k1} - \dfrac{1}{k3-k2} \right) e^{-k3t} \right) + A_0$