tisAB Standby Site and Translation Efficiency

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Summary

Translation initiation is the key step in post-transcriptional regulation in bacteria, and the ribosome binding site (RBS) is the hot spot in the competition between the stable secondary structure of mRNA with its unfolding and binding to 30S ribosomal subunit. However, the recently discovered istR-tisAB system in *Escherichia coli* K12 has provided another example of a ‘standby’ model, which postulates initial binding of ribosomes to a region outside the RBS. Three mRNA species, tisAB+1, tisAB+42, and tisAB+106 were discovered in vivo, in which only tisAB+42 is translationally active. The structured RBS of *tisB* sequesters it from translation, while the unstructured 5’-end region upstream of the RBS in tisAB+42 mRNA serves as the ‘standby site’, which favors translation. In this project, the ‘standby site’ from tisAB+42 and the structured 5’-domain from tisAB+1 were respectively cloned into a vector containing a repA-lacZ fusion. Results from both blue-white screenings and beta-galactosidase assays showed that the expression level of the construct containing the 5’-ends of tisAB+42 was as low as that containing the 5’-tisAB+1 segment, which indicated that the tisAB standby site did not show significant enhancement of the translation efficiency in the repA-lacZ system. One possibility might be that the RNAs were not fully expressed. Further work such as Northern-blot and reverse-transcription-PCR is needed to check the products at the RNA levels if there were any, and more constructs such as the one containing 5’- tisAB+106 segment or mutations in the potential ‘standby site’ are to be made to test the ‘standby activation’ hypothesis.
Introduction

Translation Initiation
Translation, the process of mRNA-encoded protein synthesis, requires a complex machinery with ribosomes, transfer RNAs (tRNAs) and protein factors involved (Marintchev & Wagner, 2004), as well as the interplay of messenger RNA (mRNA) structure and its binding to ribosomal subunits (Kaberdin & Bläsi, 2006). Among all major stages (initiation, elongation, termination and recycling) of translation, initiation is the most complicated and best regulated. It is also the rate-controlling step.

The ribosome, the protein-synthesizing ribonucleoprotein particle, functions through ligand recognition (binding of mRNA, tRNA and translation factors) and catalysis (peptidyltransferase activity). Oscillations between open (unlocked) and closed (locked) conformations are proposed to be necessary events in the processes of aminoacyl-tRNA binding, transpeptidation and translocation (Spirin, 2004). The intact ribosomes are 70S (consisting of the 30S small subunit and 50S large subunit, S: sedimentation coefficient) in bacteria and archaeb, and 80S (40S+60S) in eukaryotes (Marintchev & Wagner, 2004).

The translation initiation region (TIR) of the mRNA and the initiator tRNA (fMet-tRNA\(^\text{fMet}\)) are selected by the ribosome to form a ternary complex, known as the initiation complex (IC). Its formation is kinetically controlled by three translation initiation factors (IFs), IF1, IF2 and IF3 (Gualerzi & Pon, 1990). There are four major tasks performed by the translation apparatus during the initiation: 1) subunit dissociation and anti-association, 2) selection of the initiator aa-tRNA, 3) selection of the correct translation start site, and 4) subunit joining at the start codon (Marintchev & Wagner, 2004).

Regulation of Translation Initiation
Bacterial mRNAs have a conserved motif called Shine-Dalgarno (SD) sequence, around 5–12 nucleotides (nt) upstream from the start codon. Upon formation of the IC, the complementary anti-SD sequence residing at the 3’ end of 16S rRNA in ribosomal 30S subunit will bind to the single-stranded region of SD on mRNA (Shine & Dalgarno, 1974). This ribosome binding site (RBS) is crucial in translation. The anticodon of fMet-tRNA\(^\text{fMet}\) recognizes the start codon, which is generally AUG, with GUG, UUG or AUU as possible alternatives (reviewed in Jacques & Dreyfus, 1990) at the start of the open reading frame (ORF). Then IC scans along the mRNA molecule, and translation continues. There are several regulatory mechanisms, including the rearrangement of mRNA secondary structures, riboswitches and translational coupling.

Rearrangement of mRNA Secondary Structure & Riboswitches
The intra-molecular base-paring of mRNA may compete with its binding to the ribosome. The ribosome has to overcome the thermodynamic barrier of mRNA secondary structure to form a stable mRNA:30S complex (Studer & Joseph, 2006). de Smit & van Duin (1990) pointed out because: 1) the non-specific initial interaction for a large part is handled by S1 , which is an single-stranded RNA-binding protein in the ribosomal small subunit; 2) SD-antiSD base-pairing only can occur when the SD sequence is unpaired; 3) also the start codon should not be base-paired, to allow interaction with the anticodon of
fMet-tRNA<sup>fMet</sup>, only a locally single-stranded form of mRNA is open to access the ribosome in initiation (Figure 1, upper panel). However, if the mRNA forms a stable secondary structure such as in double-stranded or stem-loop conformations, the 30S subunit will be prevented from binding to the RBS, and in that case translation is inhibited (Figure 1, lower panel).

![Figure 1: Effect of mRNA secondary structures on translation. The ribosomal 30S subunit binds to the single-stranded RBS, and translation initiates (upper panel), while the structured mRNA prevents ribosome binding and in that case translation is inhibited (lower panel).](image)

A good example of gene expression regulation through modulation of the secondary structure of mRNA is ‘riboswitch control’ discovered in recent years (reviewed in Nudler & Mironov, 2004). Riboswitches, also known as ‘RNA sensors’, are segments of the 5’-untranslated region of certain bacterial mRNAs that encode certain metabolic proteins. Specific ligands (e.g. amino acids, nuclear bases and sugars etc) bind directly to the riboswitches, leading to rearrangement of secondary structure and modifying the expression of biosynthetic or transport proteins for those ligands (Coppins et al., 2007).

**Translational Coupling**

Translational coupling is another approach found in bacteria, with the most illuminating examples of the coat and lysis genes of RNA phage MS2 (Schmidt et al., 1987) and the tap-repA genes in plasmid R1 (Blomberg, 1992). The repA-mRNA-encoded RepA is the rate-limiting protein for the plasmid’s replication initiation. Due to the stable stem loop structure in the RBS region, repA cannot be translated from the naked mRNA. There is a translational activator peptide named Tap composed of 24 amino acids, translated upstream of RepA. A possible function of tap is suggested by the overlap between its stop codon and the RBS region of repA (Figure 2). In this case, ribosomes terminating at the tap stop codon could reinitiate at the nearby repA start codon (direct translational coupling), so that RepA is expressed (Blomberg, 1992).
‘Activation by Standby’ Hypothesis
Investigations of the coat-protein gene from bacteriophage MS2 (de Smit & van Duin, 1990, 1994) have demonstrated that, the stronger the hairpin is, the less the 30S subunit can compete, and as a result, the lower expression becomes. In the case of stable hairpin in the coat gene RBS, calculations indicated that the time window during which the folded RBS is open is generally much too short to recruit a free 30S subunit from the cytoplasm. To achieve efficient expression, a 30S subunit may already be in contact with the mRNA while it is still folded, await the opening of the structure, and subsequently ‘slide’ into place and be fixed by the SD and codon-anticodon interactions. Single-stranded regions flanking the structure may constitute a ‘loading pedal’ or ‘standby site’, to which the 30S subunit can attach non-specifically (de Smit & van Duin, 2003).

Recently, the ‘standby model’ has gained experimental support through fluorescence resonance energy transfer (FRET) studies (Studer & Joseph, 2006). The formation of IC on a short, stable RBS-containing hairpin did not occur without a preceding single-stranded stretch of nucleotides. Because all RNA is structured to some degree, standby binding may be a general feature of translational initiation.

The tisAB System and Standby Site
Since the first two natural antisense RNAs were discovered around half a century ago: RNA I in plasmid ColE1 (Tomizawa et al, 1981) and CopA in plasmid R1 (Stougaard et al, 1981), research on regulation mediated by antisense RNAs has witnessed great advances during the past few decades. A first antisense RNA-regulated SOS-induced toxin was recently described in *E. coli* K12 (Vogel et al, 2004). This SOS-induced toxin functions to arrest the cell growth under DNA damage conditions, to provide the bacteria time to recover and carry out DNA repair. The *istR-tisAB* locus (*istR*: inhibition of SOS induced toxicity by RNA, *tis*: toxicity induced by SOS) is shown in Figure 3 (Vogel et al, 2004, Darfeuille et al, 2007).

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Figure 2: Model of tap-repA translational coupling. The Shine-Dalgarno (SD), stop codon of *tap*, and start codons of *tap* and *repA* are indicated. Without translation of *tap*, the stable RNA stem-loop that sequesters the *repA* RBS inhibits translation of *repA*. With *tap* translation, ribosomes terminate at the *tap* stop codon, and can reinitiate at the *repA* RBS by direct translational coupling. Reproduced from Kolb *et al*, 2000 with permission from the copyright holder.
The transcription of *tisAB* under LexA control is induced by DNA damage and SOS response. A toxin peptide named TisB is encoded by *tisB* (*tisA* is not involved). Two small antisense RNAs, IstR-1 (constitutively synthesized) and IstR-2 (LexA controlled), are transcribed from two leftward promoters, and they share identical 3’-ends. Under normal growth conditions, the complementary stretch of 21 nt in the 5’-tail of IstR-1 base-pairs rapidly with its target region near the *tisA* start codon, around 100 nt upstream of the *tisB* RBS, thereby the synthesis of toxin TisB is inhibited. IstR-2 does not take part in *tisAB* regulation (Vogel *et al.*, 2004; Darfeuille *et al.*, 2007).

Three *tisAB* mRNA species were observed in vivo (Vogel *et al.*, 2004): *tisAB*+1, the full-length primary transcript; *tisAB*+42, produced via endonucleolytic processing (by an as yet unidentified endoribonuclease) 42 nt from the 5’-end of *tisAB*+1; and *tisAB*+106, resulting from IstR-1 binding and subsequent RnaseIII cleavage. All of the three share identical 3’-end part, but the 5’-ends differ from each other (the secondary structures are shown in Figure 4). Only *tisAB*+42 is translationally active, but not *tisAB*+1 and *tisAB*+106 (Darfeuille *et al.*, 2007).

One feature that could explain its translatability is a long weakly structured 5’-end present only in *tisAB*+42, which could serve as a "standby site" that is open and accessible to free 30S subunit (Figure 4). The full-length *tisB* RBS is highly structured, and as a result translation initiation is prevented. The model (Darfeuille *et al.*, 2007) in Figure 5 proposes that the ribosomes loaded on the single-stranded region help to translate from the structurally blocked RBS: upon breathing of the RBS structure, standby ribosomes slide into the transiently open structure, and translation starts.
Figure 4: Schematic representation of secondary structures for tisAB+1, +42 and +106 mRNAs (reproduced from Darfeuille et al, 2007, with permission form the copyright holder). The secondary structure model of the entire tisAB+1 is shown. Nucleotides encircled in gray show the IstR-1 target sequence (tisA ORF AUG in red letters). The uniform 3’ domain is highlighted in light blue. The tisB SD and AUG are indicated as blue circles, and the UAA stop codon in dark red. For tisAB+42 and +106 mRNAs, only the different 5’ regions and the first stem loop of the downstream part are shown.

Figure 5: Standby activation model (reproduced from Darfeuille et al, 2007 with permission form the copyright holder). Simplified structures are based on Figure 4, with translationally active and inactive states of the mRNAs indicated.
How to Monitor the Translation Efficiency: LacZ Reporter

Translation efficiency in vivo can be monitored by a LacZ reporter system. As a result of the translational fusion, expression of the target genes can be assessed indirectly via measuring the activity of beta-galactosidase, the product of the lacZ gene.

*Semi-quantitative Test of Beta-galactosidase Activity*

Beta-galactosidase is encoded by lacZ of the lac operon in *E. coli*. It is a 464-kDa tetramer of four identical 1023-amino acid chains (Fowler & Zabin, 1970; Matthews, 2005). The enzyme's function in the cell is to hydrolyze the disaccharide lactose into the monosaccharides glucose and galactose, so that they can be used as carbon/energy sources by the bacteria. Alternative names are "beta-gal" or "β-gal", and lactase is a subclass of the beta-galactosidase.

In gene cloning, the X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) assay is used to indicate whether a bacterium expresses beta-gal activity, and it has another name of “blue-white screening”. X-gal is hydrolyzed by this enzyme, yielding galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is further oxidized into 5,5'-dibromo-4', 4'-dichloro-indigo, which is an insoluble dark blue compound (Horwitz et al, 1964; Davies & Jacob, 1968). Thus, if X-gal and an inducer of beta-gal (usually IPTG: 1-isopropyl- β-D-thiogalactopyranoside) are contained within an agar medium, colonies that have a functional lacZ gene can easily be distinguished by their blue color. Those without LacZ expression will form white colonies on the culture plate.

*Quantitative Measurement of beta-galactosidase activity*

The compound ortho-nitrophenyl-beta-D-galactoside (ONPG) is also recognized as a substrate of beta-gal and is cleaved to yield galactose and ortho-nitrophenol (ONP) (which has yellow color and absorbs at a wavelength of 420 nm). When ONPG is in excess over the enzyme in a reaction, the production of ONP per unit time is proportional to the concentration of the enzyme. Thus, the activity of beta-gal can be quantitatively monitored (http://openwetware.org/wiki/Beta-Galactosidase_Assay_(A_better_Miller) by reading out the accumulation of the yellow product of ONP under a spectrophotometer.

In 1972, Jeffrey Miller published "Experiments in Molecular Genetics", which gave a protocol for determining the amount of beta-gal with ONPG (Miller, 1972). For this reason, ONPG/beta-gal assays are also referred to as "Miller assays", and a standardized amount of beta-gal activity is defined as a "Miller Unit". 1 Miller Unit = 1000 * (OD420 - (1.75*OD550))/t*v*OD600, where: OD420 is the absorbance of the yellow o-nitrophenol, OD550 is the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420 nm, t is the reaction time in minutes, v is the volume of culture assayed in milliliters, and OD600 is a measure of the cell density.

Combination of the *tisAB* and *repA-lacZ* system

The entire *tisAB* mRNA can be divided into two modules. The first one can be described as an ‘activating part’ (containing the ‘standby site’), and the second contains the reading frame that cannot be translated (Darfeuille et al, 2007). This activating part will be cloned into the upstream region of *repA* of plasmid R1, which has been fused to a lacZ reporter.
gene. As aforementioned, without tap coupling, translation of repA does not occur due to a strong secondary structure around the RBS.

**Aim of this Project**
The aim of my project was to ask whether the "activating" 5'-half of tisAB mRNA could increase repA expressions. Measurements and comparison of the translation efficiencies were carried out by X-gal tests and ONPG/beta-gal assays.
Results

Construction of Plasmids

In order to construct the target plasmids pLL01, pLL42 and pLL106 (Table 3), plasmid vector pCU10 was made from the original pGW177-L (Figure 7, left panel). All the constructs were confirmed by sequencing.

Figure 6: Schematic picture to show the construction of plasmid pCU10 (left panel) and the tisAB inserts in pCU10. The restriction sites were shown, and lacZ gene was boxed in green.

The repA gene in plasmid pGW177-L (Blomberg et al., 1992) is translationally fused to the lacZ reporter gene (Figure 6), so that repA-lacZ expression can be measured by beta-galactosidase assay. The restriction endonucleases Bgl-II and Sal-I were used to cut at two ends upstream of the repA promoter and the site of lacZ fusion, separately (Figure 7, left panel). The ORF of tap remained intact, and the stop codon of tap was situated downstream of the repA RBS, therefore ribosomes reading through the tap can reinitiate at the repA RBS nearby.

Figure 7: Left panel: Part of the sequence of plasmid pGW177-L. The antisense strand (complementary strand) is colored in green. The restriction sites for Bgl-II and Sal-I are highlighted with red letters, with the names of endonucleases indicated above the spots. The Nco-I cleavage site was introduced via PCR mutagenesis, by the primer of repA prom rev.
The repA SD, and repA start codon as well as tap stop codon are marked in blue. The primers of ‘repA prom rev’ and ‘repA fw’ were designed to be complementary to each other. The truncated part between these two primers to construct pCU10 includes the SD sequence and start codon of tap. In pGW177-L, with the translational coupling to tap, ribosomes reading through tap ORF can reinitiate at repA RBS, and thereby repA is expressed (more detailed refer to the text). Right panel: Part of the sequence of pCU10.

To construct plasmid pCU10, the ‘repA prom rev’ primer sequence was designed to be complementary to the sequence in the primer of ‘repA fw’, thus these two parts could base pair with each other. After annealing and filling in, the region between these two primers in pGW177-L was deleted, including the SD sequence and start codon of tap truncated as well (Figure 7, right panel), and this made a non-functional tap. The repA ORF was intact but repA SD and start codon were part of a stable stem-loop structure (Figure 2). In this case, repA cannot be translated without the intact tap. An Nco-I cleavage site at the repA transcription start site was introduced by PCR mutagenesis, in order to be able to insert the segments from tisAB, to make pLL01, pLL42 and pLL106.

Finally 150 nt of the 5’-ends in tisAB+1 and 109 nt in tisAB +42 were successfully inserted into the region upstream of the repA RBS, resulting in the constructions of pLL01 and pLL42 (Figure 6, right panel). In plasmid pLL01, the structured 5’-domain from tisAB+1 (which is composed of a strong structure of three loops and a stable double-stranded stem followed by another loop) was ligated to the repA RBS (Figure 8, left panel); and the relatively weak-structured end of tisAB+42 (with bulges and short double-stranded stems including unstable U-G pairs, and a stretch of 15 nt single strand plus the downstream loop) was inserted in pLL42 (Figure 8, right panel), serving as the potential ‘standby site’. Unfortunately, due to the limited time and pause of sequencing service during the summer vacation from the genome centre, up to the date the report was written, the construction of pLL106 has not been accomplished yet.

Figure 8: Schematic representation of pLL01 (left panel) and pLL42 (right panel) construction. Only the insertion of tisAB part and the repA RBS are shown (from Unoson, unpublished work).
Semi-quantitative Monitor the Translation Efficiency

To semi-quantitatively monitor the beta-galactosidase activity in the target plasmids, ‘blue-white screening’ was done. The colonies of pGW177-L (positive control) were dark blue on the X-gal agar, while colonies of pCU10 (negative control) and pLL01 & pLL42 (test plasmids) were white color (Figure 9). This indicated that there were no significant differences in beta-galactosidase activities among pLL01, pLL42 and pCU10.

![Figure 9: picture of 16-hour-incubated culture, with X-gal on LA+Kanamycin plate.](image)

Quantitative Measurement of the Translation Efficiency

In order to compare the different repA-lacZ expression levels in pGW177-L, pCU10, pLL01 and pLL42 accurately, a quantitative measurement was carried out by ONPG/beta-gal assay. Three independent cultures were made for each construct (i.e. three samples for each plasmid), with two duplicates of each sample. The Miller Units for pGW177-L were as high as 375.10±45.36 (for raw data, see Appendix), while the absolute values for pCU10, pLL01 and pLL42 were close to zero and the beta-galactosidase expression levels from these strains were too low to be detectable.

The conclusion from beta-gal assays was in accordance with the results from the blue-white screening, that is: no significant enhancement of repA-lacZ expression was observed in plasmid pLL42, compared to pLL01 and the negative control pCU10 in this experiment.
Discussion

The Legitimacy of Standby Model

According to de Smit & van Duin (1990), the binding of S1, the interactions between SD-antiSD and codon-anticodon all require the mRNA to be locally single-stranded. However, while an RBS may in general be less structured than other mRNA parts (Ganoza et al., 1987), it is seldom structure-free.

The folding rate of the MS2 coat-gene RBS is predicted to be around $10^5$ s$^{-1}$, which implies an average lifetime of the unfolded state of $1/10^5$ second = 10μs. Given the limited diffusion rates of ribosomes and free ribosomes’ concentration in the cytosol (~8.5 μM as upper limit) (de Smit & van Duin, 2003), a free 30S subunit will on average take 4 ms to collide with the RBS (de Smit & van Duin, 2003). It appears that the cytoplasm-dispersed ribosomes are simply too slow to jump onto the RBS within the brief instant that it is accessible. Since translation of the coat gene is not coupled to any other genes present on the MS2 RNA, the ribosomes that initiate at the coat start might sit somewhere on the RNA, waiting for the RBS to become available. Standby binding therefore might provide a satisfactory solution to the kinetic paradox.

The 30S ribosomal subunit can bind to the messenger covering 35-50 nt (Hüttenhofer & Noller, 1994). In the tisAB system, experimental data demonstrated that the ‘activating part’ of a weakly structured 5’ end and a following single stranded region of 15 nt (and this amount to more than 35 nt together) in tisAB+42 mRNA favored the translation, serving as the standby site (Darfeuille et al., 2007). Then why did this part not work, when inserted into the repA-lacZ system?

Possible Explanations For the ‘Null Standby Site’ in this work

My results from both the blue-white screening and beta-gal assays showed that the standby site from tisAB mRNA did not significantly enhance the translation efficiency of repA-lacZ as expected, or, the standby site was ‘null’ in this construct. There could be several possibilities:

The RNAs of pLL01 and pLL42 were not fully expressed in the constructs, or they were unstable and subsequently degraded. If this was the case, the ‘potential standby site’ would not exist in pLL42, and as a result its repA-lacZ fusion would not have higher translation efficiency than the full-length construct.

If the RNAs were expressed, then its meaning is not obvious. First of all, only the sequences (primary structure) of my plasmids were checked, but the secondary structure of the potential ‘standby site’ in pLL42 was not 100% certain before further experimental determinations. It can be somehow different from that in tisAB+42 mRNA, and even slight differences can account for the ‘non-working’ standby site.

Secondarily, other factors, such as the tertiary structure of the target mRNAs or other ‘neighboring sequences’ surrounding them can affect the translation efficiency. There are no descriptions of an influence of 3D-structures on translation in the literature; however,
as the tertiary structures of both DNA and proteins have shown to be related to their biological functions and mechanisms, it is indeed possible that mRNA’s tertiary conformation can have something to do with its translation. And the sequences near to the target region on the messenger may interplay with it to form a yet-unknown structure, which may destroy the ‘standby site’ as well.

Finally, as environmental factors evidently can affect the translation efficiency, the physiological conditions in the cells, such as pH, salt concentration and so on, might have effects on translation but the significance of the influences is not exactly sure yet.

Future Work to Do
To further test this tisAB ‘activation by standby’ model in the constructed repA-lacZ system, the following can be done:

1) Do Northern blot or reverse-transcription PCR (RT-PCR), to check the expressions of pLL01 and pLL42 on RNA level. Either by displaying the RNA products on the membrane, or amplifying the complementary DNA (cDNA) of the target RNAs via RT-PCR, it will be able to see whether the RNAs have been expressed.

2) If no RNA expressions are found in pLL01 and pLL42, other systems instead of the repA-LacZ reporter can be tried. If the standby site from tisAB+42 can work in the new system, continue with construction of plasmid containing the 5’-end from tisAB+106, and another transformation with the small antisense RNA IstR-1 in plasmid containing the tisAB+42 standby site. As mentioned in Darfeuille et al, the standby structure is only existed in tisAB+42 mRNA, but not available in tisAB+1, tisAB+106 or IstR-1-bound tisAB+42: the stable, long 5’ domain of tisAB+1 may resist unfolding, and the short 5’ end of tisAB+106 would be insufficient to bind 30S; and in tisAB+42, once bound with IstR-1, the ribosomal loading trace is occupied and 30S is excluded. Comparison of the translation efficiencies in these different constructs will further shed the light on the mechanism of regulation.

3) Test other constructs if it works to further study the standby hypothesis. For example, to generate mutations in the “standby site”, or cut off different lengths of the nucleotide if its standby activation works, to find out the minimum length of the standby site.
Methods and Materials

Chemicals, Reagents, and Oligo-deoxyribonucleotides
Chemicals and reagents were purchased from Sigma-Aldrich or GE Healthcare unless otherwise specified. Oligodeoxyribonucleotides were from Sigma-Genosys (Table 1).

Table 1: oligodeoxyribonucleotides used for generation of \textit{tisAB-repA-lacZ} fusion

<table>
<thead>
<tr>
<th>Name of primers</th>
<th>Sequence 5’-3’</th>
<th>Tm °C</th>
<th>Size (nt)</th>
<th>Comments</th>
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<td>TTGTGCAGCAACACAGATCT AATGCGCAGAAGGAGCGAG</td>
<td>60</td>
<td>20</td>
<td>Underline part: for Bgl-II enzyme</td>
</tr>
<tr>
<td></td>
<td>CATGGGTAAATCTAGCATGC</td>
<td>60</td>
<td>19+20</td>
<td>Underline part: for Nco-I enzyme</td>
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<td>20+19</td>
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<td>repA fw</td>
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<td>60</td>
<td>19</td>
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<tr>
<td>repA rev</td>
<td>AATGCGCAGAAGGAGCGAG CCATGGGTAAATCTAGCATGC</td>
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<td>12+20</td>
<td>\textit{tisAB} +1 including Nco-I site + 8 extra residues for cloning into pCU10</td>
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<tr>
<td>3’-tisAB Nco-I</td>
<td>AATGCGCAGAAGGAGCGAG GCATCAATAGCGCGTCCGAC TAGATTACCAGGGGTACGG</td>
<td>60</td>
<td>12+20</td>
<td>\textit{tisAB} +42 including Nco-I site + 8 extra residues (for cloning into pCU10)</td>
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<td></td>
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<td>60</td>
<td>12+19</td>
<td>\textit{3’-tisAB} including Nco-I site + 8 extra residues for cloning into pCU10</td>
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Strains and plasmids
Strains of bacteria are shown in Table 2, and the plasmids shown in Table 3.

Table 2: Strains of bacteria in this project

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<td>TOP 10</td>
<td>YT media, 37 °C</td>
<td>Commercial competent cells</td>
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Table 3: Plasmids in this project

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<th>Plasmid</th>
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<th>Marker</th>
<th>Relevant properties</th>
<th>Sources or references</th>
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<td>pGW177-L</td>
<td>p15A</td>
<td>Km\textsuperscript{R}</td>
<td>Positive ctrl, with full length \textit{tap} coupling to \textit{repA-LacZ} fusion, \textit{LacZ}\textsuperscript{-}</td>
<td>Blomberg \textit{et al}, 1992</td>
</tr>
<tr>
<td>pJV975-1</td>
<td>pGW177-L</td>
<td>Km\textsuperscript{R}</td>
<td>Carries \textit{tisAB} system, serving as the DNA template to amplify the 5’-ends of three \textit{tisAB} mRNAs</td>
<td>Vogel \textit{et al}, 2004</td>
</tr>
<tr>
<td>pCU10</td>
<td>pGW177-L, pJV975-1</td>
<td>Km\textsuperscript{R}</td>
<td>Negative ctrl, with part of \textit{tap} frame truncated, \textit{LacZ}\textsuperscript{-}</td>
<td>This work</td>
</tr>
<tr>
<td>pLL01</td>
<td>pCU10, pJV975-1</td>
<td>Km\textsuperscript{R}</td>
<td>Test plasmid with \textit{repA-LacZ} fusion, including the “inactive” 5’-</td>
<td>This work</td>
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</tbody>
</table>
Test plasmid with \textit{repA-LacZ} fusion, including the “activating” 5’-end of \textit{tisAB}+42 which serves as the potential “standby site”

Test plasmid with \textit{repA-LacZ} fusion, including the “inactive” 5’-end from \textit{tisAB}+106, construction not accomplished in this project

<table>
<thead>
<tr>
<th>Steps</th>
<th>Functions</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Inactivation of contaminating enzymes</td>
<td>95 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>2.</td>
<td>Denaturing*</td>
<td>95 °C</td>
<td>40 sec</td>
</tr>
<tr>
<td>3.</td>
<td>Annealing*</td>
<td>60 °C</td>
<td>40 sec</td>
</tr>
<tr>
<td>4.</td>
<td>Elongation*</td>
<td>75 °C</td>
<td>40 sec</td>
</tr>
<tr>
<td>5.</td>
<td>Provide with extra time for elongation</td>
<td>75 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>6.</td>
<td>To keep the PCR product stable after the program</td>
<td>4 °C</td>
<td>For ever</td>
</tr>
</tbody>
</table>

*: Repeat Steps 2-4 for 35 cycles to amplify the DNA.

**: As the annealing temperatures of most primers in this project were designed to be 60 °C or near 60 °C, all the PCR programs in this project were using the same amplification condition.

**Construction of Plasmid pCU10**

An overnight culture of \textit{E. coli} plasmid pGW177-L was purified (Nucleo Spin® plasmid Macherey-Nagel mini kit) to serve as the DNA template in PCR. Primers used to generate the vector are shown in Table 1. The plasmid pU10 was generated in a two-step protocol. First, primers of \textit{repA} prom fw and \textit{repA} prom rev generated an ‘upstream’ fragment containing the promoter of \textit{repA} and site for Nco-I, and \textit{repA} fw in conjunction with \textit{repA} rev generated the ‘downstream’ one including the \textit{repA} SD, \textit{repA} start and \textit{tap} stop. After PCR purification (Qiagen™ PCR purification kit), pairs of PCR fragments (200 ng) were annealed at 85°C for 15 min (annealing buffer: 10 x Tris EDTA (TE buffer: 100 mM Tris-Cl, 10mM EDTA [pH 8.0]), 0.1 M NaCl [pH 7.5]) and cooled to 30 °C. Components were added to obtain a final concentration of 0.2 mM dNTPs, 10 x Klenow buffer (Fermentas™), and 0.15 U/μl Klenow fragment of DNA polymeraseI (Fermentas™) to generate filling in products. After incubation at 37 °C for 15 min, the DNA was amplified via PCR, using \textit{repA} prom fw and \textit{repA} rev as primers. The purified PCR products (inserts) and plasmid pGW177-L were cleaved by restriction endonucleases Bgl-II (0.6 U/μl) and Sal-I (0.9 U/μl) with 10 x buffer, at 37 °C for 1 hour, separately. After that the samples were treated with PCI purification (200 μl dH2O was added to each sample, plus 250 μl PCI (Phenol: chloroform: isoamyl alcohol=25:24:1). A phase-lock tube [in this tube, the aqueous and organic phases will be separated, with upper phase of aqueous and lower organic phase] was centrifuged (9000 x g, 1 min). Then 500 μl of the liquid was transferred to it, and mixed by strong shaking. The tubes were centrifuged for 5 min at 9000 x g. About 250 μl of the upper phase from the sample was transferred to a new eppendorf tube. Then 625 μl of 99% ice-cold EtOH and 15 μl of
3M NaAC were added and the tubes were kept in freezer at 20 °C for more than 1 hour, followed by centrifugation at 4 °C for 30 min at 9000 x g. Finally, the supernatant was removed and vacuum-centrifuged for 10 min. The pellet was resuspended in 20 to 50 μl dH2O and the DNA concentration was measured. About 10 times the amount of inserts compared to the vector were added into Ready-To-Go T4 DNA ligase tubes (Amersham Biosciences™) to a total volume of 16 μl, and incubated in a 16 °C water-bath for 2 hours. Finally the ligation products were transformed into *E. coli* TOP10 cells (commercial competent cells, Invitrogen™). The transformed cells were spread on LA (10 g/L Tryptone, 5 g/L Yeast extract, 10 g/L NaCl and 15 g/L agar, pH 7.0) + Kanamycin (50 μg/ml) plates, and incubated at 37 °C overnight. After plasmid purification (Nucleo Spin® plasmid Macherey-Nagel mini kit), the samples were sent for sequencing, with the primer of repA prom fw.

**Construct Target Plasmids of pLL01, pLL42 and pLL106**
The 5’-ends of *tisAB*+1, *tisAB*+42, and *tisAB*+106 mRNAs were amplified by PCR with the reverse primer of 3’-*tisAB* Nco-I in conjunction with the 3 different forward primers of *tisAB*+1 Nco-I, *tisAB*+42 Nco-I, and *tisAB*+106 Nco-I respectively, using plasmid pJV975-1 that contains the *tisAB* system (Vogel *et al.*, 2004) as the DNA template. Then all the PCR products (inserts) and pCU10 (vector) were cleaved by Nco-I (Fermentas™) with 10 x H buffer (Fermentas™), at 37 °C for 1 hour. After heat inactivation at 65 °C for 20 min, the endonuclease-cleaved samples were treated with PCI purification as aforementioned, dried via vacuum-centrifugation, and redissolved in 20-50 μl water. In order to avoid self-ligation, pCU10 was dephosphorylated by 0.02 U/μl shrimp alkaline phosphatase (SAP, Usb®) and 10 x R buffer (Usb®), at 37 °C for 1 hour, followed by heat inactivation at 60 °C for 15 min. The ligation and transformation were done in the same way as the construction of pCU10.

**Competent Cells**
An overnight culture of *Escherichia coli* strain MC4100 were grown in LB medium (10 g/L Tryptone, 5 g/L Yeast extract, and 10 g/L NaCl, pH 7.0) at 37 °C to OD₆₀₀ 0.4-0.5 and then harvested, centrifuged at 4200 x g, 4 °C for 10 min. Then the supernatant was removed and the pellet was resuspended in 10 ml of ice-cold 0.1 M CaCl₂, centrifuged as before and re-centrifuged in ice-cold 0.1 M CaCl₂. After that, the supernatant was poured off and the remaining liquid was resuspended and the cells were ready for transformation.

**Transformation**
2 μl ligation product was added to 50 μl competent MC4100 cells and kept on ice for 30 min, followed by 42 °C heat-shock for 30 sec. Then the tubes were put on ice for 3 min again. Around 250 to 500 μl of 37 °C pre-warmed 2 x YT (yeast extract tryptone medium: 16 g/L Tryptone, 10 g/L Yeast extract, and 5 g/L NaCl, pH 7.0) was added into the tubes and the tubes were incubated at 37 °C on the shaker for 1 hour. After that, the cells were spread on LA + Kanamycin (50 μg/ml) plates and incubated overnight at 37 °C.
Blue-White Screening
40 μl of X-gal (40 μg/ml) was spread on LA + Kanamycin (50 μg/ml) plates and dried on the bench for 1 hour. Then single colonies from pGW177-L, pCU10, pLL01 and pLL42 were picked and streaked on the agar. Plates were wrapped with foil, as X-gal is light sensitive. The plates were incubated at 37 °C overnight (approximately 16 hours) and the colors were monitored the next day.

Beta-Galactosidase Assays
Overnight cultures were prepared and grown in LB medium at 37 °C until OD₆₀₀ = 0.5, then 1 ml of the culture was transferred to an eppendorf tube and put on ice. The culture was pelleted at 9000 x g and 4 °C for 1 min, then the supernatant was decanted and the pellet was kept on ice again. Z-buffer (0.06 M Na₂HPO₄·7H₂O, 0.04 M NaH₂PO₄·H₂O, 0.01 M KCl, and 0.001 M MgSO₄, pH = 7) including 0.05M beta-mercaptoethanol (β-ME) was added to the tube to resuspend the cells to 1.0 OD₆₀₀/ml. 75 μl chloroform and 50 μl 0.1% SDS were added to each tube, and the tubes were vortexed 15 sec and put back on ice. Several dilutions of the samples were made by diluting in Z-buffer. After that, 200 μl Z-buffer was added in two wells in a microtiterplate for blanking. 200 μl of each sample and its dilution was loaded in the wells with 40 μl ONPG (4 mg/ml) solved in buffer (0.06 M K₂HPO₄, 0.033 M K₂HPO₄, 0.008 M (NH₄)₂SO₄, and 0.002 M Na-citrate·H₂O, pH = 7), and the Titertek spectrophotometer was set to measure at dual wavelengths at 414-540 nm. The absorbance was monitored at pre-set time points (e.g. every 5 min during 30 min). Between measurements, the microtiterplate was put in the 28 °C shaker. Finally, the activity was registered from the slopes (milli-OD/min) and Miller units were calculated in a simplified formula as follow.

Miller units = \frac{\text{SLOPE (milli-OD/min)}}{\text{OD}_{600}/\text{ml} \times \text{ml extract measured}}

The slope is automatically calculated by the kinetic program in the spectrophotometer, and it equals to 1000 * (OD₄₂₀ - (1.75*OD₅₅₀))/t.
Acknowledgements

I would like to say THANK YOU to Prof. Gerhart Wagner, for welcoming me into this exciting project, the great supervision & inspiration to my work. Filled with wisdom, humor, music (and smoke: P), you are a scientist & teacher whom I admire indeed.

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Last but not least, deep love to my dear parents and always-supportive boyfriend Junfeng Wang in China, those who have made the backbone and source of strength to me when I am far away from home. Thanks to the beautiful country Sweden, during the year I grow mature. I will miss this period of study life and the friendly people here for ever.
## References


de Smit MH, van Duin J. Translational standby sites: how ribosomes may deal with the rapid folding kinetics of mRNA. J Mol Biol. 2003, 331:737-743.


### Appendix

**Table a1: Raw data of beta-gal assay**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Dilutions</th>
<th>Slope (milli-OD/min)</th>
<th>Intercept (milli-OD)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGW177-L*</td>
<td>1 x</td>
<td>72.194</td>
<td>275.228</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68.045</td>
<td>270.085</td>
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<tr>
<td></td>
<td></td>
<td>70.862</td>
<td>332.371</td>
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<tr>
<td></td>
<td></td>
<td>65.434</td>
<td>413.561</td>
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<tr>
<td></td>
<td></td>
<td>70.017</td>
<td>300.276</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>69.748</td>
<td>344.132</td>
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</tr>
<tr>
<td>pGW177-L*</td>
<td>10 x</td>
<td>20.583</td>
<td>30.712</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
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<td>18.834</td>
<td>27.569</td>
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<tr>
<td></td>
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<td>21.651</td>
<td>28.854</td>
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<tr>
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<td>16.303</td>
<td>26.045</td>
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<tr>
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<td>19.194</td>
<td>28.902</td>
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<tr>
<td></td>
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<td>15.966</td>
<td>19.760</td>
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<tr>
<td>pCU10</td>
<td>1 x</td>
<td>0.121</td>
<td>103.651</td>
<td>0.491</td>
</tr>
<tr>
<td></td>
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<td>-0.171</td>
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<td></td>
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<td>-0.411</td>
<td>123.143</td>
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<tr>
<td>pLL01</td>
<td>1 x</td>
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<td>117.714</td>
<td>0.714</td>
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<td></td>
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<td>-0.860</td>
<td>127.700</td>
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<td></td>
<td>0.663</td>
<td>105.543</td>
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<td>pLL42</td>
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<td>136.700</td>
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<td>124.324</td>
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<td>-0.920</td>
<td>127.667</td>
<td>0.957</td>
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<tr>
<td></td>
<td></td>
<td>-1.114</td>
<td>130.214</td>
<td>0.915</td>
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</tbody>
</table>

*: The data of 10 x dilution of pGW177-L were used to calculate the final Miller Units, as the beta-galactosidases in the undiluted samples were saturated and inaccurate to be measured for the enzyme activity.