A Thesis
for the Degree of Master of Science

Construction of efficient expression vector system using *cis*-acting elements for *Lactococcus lactis*

Cis-acting elements를 이용한 유산균용의 효율적인 발현 벡터 시스템 구축

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농 학 석 사 학 위 논 문

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이 논문을 농학 석사학위논문으로 제출함

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Summary

Many approaches have been attempted to improve the heterologous protein in various bacteria. Specifically, the plasmid-based expression system has been used to achieve the recombinant protein production as an easy and useful tool to manipulate. There are three strategies to improve the expression level of recombinant protein. Such as 1) introduction of high copies plasmid-based expression vector system, 2) construction of gene multimerization cassette as a insert and ligation with the backbone vector, and 3) search of new strong promoter. However there are several limitations for these strategies in that it is hard to replicate the DNA, is too large to transformed which causes genetic instability, and is hard to predict promoter strength. In this study, I modified the promoter region and tested on the promoter strength. In addition, I tried to introduce another *cis*-acting elements such as a transcriptional terminator and RBS (ribosome binding site) to improve the expression of recombinant protein.

Lactococcus lactis subsp. lactis IL1403 is widely used in the dairy and animal industries, and it is also studied for a live oral vaccine product to elicit mucosal immune response. The translational elongation factor Tu (*tuf*) gene is a house-keeping gene, and *tuf* promoter is characterized as a strong promoter in IL1403. In this study, *tuf* promoter was modified to test the efficiency of protein expression using the luciferase gene as a reporter.

Firstly two terminators, T*rrnB* and T*pepN* were tested for the luciferase gene expression efficiency. T*pepN* terminator showed

better performance in luciferase expression.

Next, series of *tuf* promoter modification were attempted. In bacteria, RNA polymerases and several sigma factors recognized and recruited approximately -35 and -10 region upstream from the transcription start site. The core region including -35 and -10 hexamers in *tuf* promoter (119 bp) was amplified and series of modified *tuf* promoters were constructed using PCR with partial complementary reverse primer. There PCR products (#1) were cloned into the promoterless pIL.Ptuf.Luc(X) vector. Luciferase activity of t2, t4, t6 and t7 were higher than control *tuf* promoter. Especially t2 and t4 showed better performance, thus selected for next experiment.

It is well known that the sequence between RBS and start codon (ATG) are important for protein translation efficiency. Thus, I modified original sequence of this region, 'CATTTTTCAT' to 'AATTTTTAAA' to give more AT-rich. This modification was combined with selected modified *tuf* promoter to give a series of new *tuf* promoter cassette. The transformed IL1403s containing modified promoter (#2) were assayed for luciferase activity. Derivative of t2 and t4, t2-1 and t4-1 showed better performance.

Combined all the modified clones, luciferase activity was compared. t4-1 showed much higher activity compared to the t4, indicating the sequence between RBS and start codon is important for protein expression. To confirm this results, luciferase expression was analyzed on SDS-PAGE and western blot assay. Luciferase bands (61 kDa) was not detectable in SDS-PAGE, but in western blot, clones with t2, t4, t4-1 showed stronger signal compared to original *tuf* one.

In conclusion, this study revealed that introduction of modified strong promoter and additional *cis*-acting elements can improve the protein expression in IL1403. And this strategy has a prospect to improve recombinant protein expression. Since pIL252 is a low copy plasmid-based expression system, high copies-based plasmids are needed to increase recombinant protein expression.

Key words: Lactic acid bacteria, *cis*-acting element, promoter modification, *tuf*

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List of Abbreviations

BCA: Bicinchoninic acid
bp: base pair
BSA: Bovine serum albumin
cat: Chloramphenicol acetyltransferase gene
CIAP: Calf intestinal alkaline phosphatase
DNA: Deoxyribonucleotide triphosphate
ECL: Enhanced chemiluminescence
erm: Erythromycin
GALT: Gut associated lymphoid tissue
GDP: Guanosine diphosphate
GIT: Gastrointestinal tract
GTP: Guanosine triphosphate
HRP: Horseradish peroxidase
IgG: Immunoglobulin G
kDa: Kilo dalton
LAB: Lactic acid bacteria
L. lactis: *Lactococcus lactis*
Luc: Luciferase
M: Marker
NICE: Nisin-inducible controlled gene expression
OD: Optical density
PAGE: Polyacrylamide gel electrophoresis
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
RBS: Ribosome binding site
RLU: Relatively light units
RNA: Ribonucleic acid

SDS: Sodium dodecyl sulfate

T*pepN*: Terminator of *pepN* gene

T*rrnB*: Terminator of *rrnB* gene

I. Introduction

Lactococcus lactis (*L. lactis*) is known as GRAS 'generally recognized as safety' and non colonizing lactic acid bacterium. *L. lactis* IL1403 is the best-characterized *L. lactis* strain and widely used for an efficient expression vector system. Recently, IL1403 is developed as a safe live oral vaccine for the induction of mucosal immune response. But the expression of recombinant protein in IL1403 is usually low. Thus, the goal of this study is to construct expression vector system for IL1403.

The plasmid-based expression system is widely used as an easy and useful tool to manipulate for high recombinant gene expression. There are some general strategies for increasing the recombinant protein expression including high copy plasmid-based expression system, gene multimerization and introduction of strong promoter. However, there are several problems in high copy plasmid-based expression system and gene multimerization since it is hard to replicate and transform, and is genetically unstable, suggesting that it is needed for ar strong promoter. For strong promoter selection, reporter gene without promoter was used to isolate the promoter for the target host, or select the known strong promoter. But selected promoter strength in target host strain may differ from the original host. In this study, I tried to modify *tuf* promoter which known as a strong promoter in IL1403 (Kim et al. 2009). And additional *cis*-acting elements such as a transcriptional terminator and ribosome binding site (RBS) were tested for the reporter gene expression.

To validate the ability of the modified promoters, the luciferase gene was cloned as a reporter gene from pGL3-basic vector system. For terminator sequences, a short terminator (23 bp),

T*rrnB,* and T*pepN* were studied for the efficient transcriptional termination.

And *tuf* promoter was joined side by side to multimeric form to test the promoter strength in recruiting RNA polymerases and sigma factors. And some sequence variation form of modified promoters were also included in the study. All modified promoters were evaluated by luciferase assay and the promoter activity was compared by SDS-PAGE and western blot.

II. Review of Literature

1. Lactic Acid Bacteria (LAB)

1) Lactic Acid Bacteria

Lactic acid bacteria (LAB) belong to the gram positive bacteria which includes *Streptococci, Lactococci* and *Lactobacilli* (Roshan et al., 2011). Several LAB are characterized in Table 1. LAB dwell in the intestine of animal and human (Daniela et al., 2011) which can produce lactic acid from lactose fermentation. So many traditional and fermented production of food, and beverages are associated with LAB (Teuber and Geis, 2006).

Table 1. Differentiation of the LAB.

Genus	Gram stain Morphology	Type of Lactic Acid formed
Streptococcus	Cocci/chains	L^+
Pediococcus	Cocci/tetrad	DL, L^+
Lactococcus	Cocci/chains	L^+
Lactobacillus	Bacilli/pairs	D^-, L^+, DL

* L^+: levo-lactic acid
* D^-: dextro-lactic acid
* DL: D and L lactic acid

2) LAB as probiotics

In 1965, Lilly and Stillwell referred to LAB as probiotics. The probiotic was described as "a live microbial feed supplement beneficial to the host by balancing the microbial environment within its body" (Fuller et al., 1989). Food and digestive fermenters exist in the gastrointestinal tract. The mucous membranes of intestine provide the complex environment which live the 10^{14} bacteria of different species (Savage et al., 1998). And microflora plays a crucial role in the gastrointestinal tract, such as the physiological and immunological response to the host. It can rapidly stimulate the mucosal immune response against to the infection of pathogens and inhibits the colonization of pathogens at the gut membranes (Cebra et al., 1999). The secretion of IgA derived from subepithelial immune cells is important in the oral vaccine delivery system (McGhee et al., 1989). The mechanisms may vary from one strain to another and are probably a combination of activities, in most cases. Thus, the definition of mechanisms is a very difficult and complex task. In general, the distinguished three levels of action are proposed. Probiotics can influence host health by interacting environments with other microorganisms, by beneficial mucosal barriers, and by stimulating the immune system (Leroy et al., 2008).

Recently, the LAB are used to stimulate the mucosal immune response as probiotics and they can produce antimicrobial products such as lactic acid and bacteriocin (Teusink and Smid, 2006). In the upper gastrointestinal tract, the population of LAB is predominant and some species such as *Lactobacillus* can colonize the mucosal membrane (Maldonado et al., 2006).

Predominant members including *Bifidobacterium spp.* and *Lactobacillus* in intestine are commonly studied as probiotics agents (Table 2). They can reduce the blood cholesterol and lactose intolerance, alleviate some intestinal diseases, stimulate the immune response and prevent the cancers (Marteau et al., 2002). Selection criteria has also been proposed including safety criteria, technological criteria, functional criteria, desirable physiological criteria. The properties of safety criteria are associated with origin, pathogenicity , infectivity and virulence factors. Technological criteria contains genetically stable strains, desired viability during processing and storage, good sensory properties, phage resistance and large-scale production. Functional criteria includes gastric acid tolerance, bile tolerance, mucosal surface adhesion and validation and documentation of health effects. The properties of desirable physiological criteria are associated with immunomodulation, antagonistic activity towards gastrointestinal with pathogens, cholesterol metabolism, lactose metabolism and antimutagenic and anticarcinogenic properties (Daoud and Hani, 2013).

Table 2. Selected organisms as probiotic agents.

Gram-positive bacteria	Human disease in which benefit is shown	Animal model in which benefit is shown
Bifidobacteria bifidum	NA	Rat model of necrotizing enterocolitis
Bifidobacteria infantis	IBS29	NA
Lactobacillus rhamnosus GG (used with lactoferrin)	Sepsis in very low birth weight infants	NA
Lactococcus lactis (engineered to produce IL-10 or trefoil factors)	Crohn's disease	DSS-induced colitis and IL-$10^{-/-}$mice
Lactobacillus plantarum 299v	Antibiotic-associated diarrhea	IL-$10^{-/-}$ mice
Lactobacillus acidophilus	NA	Visceral hyperalgesia 40 and C. rodentium-induced colitis
Lactobacillus rhamnosus	Pediatric antibiotic-associated diarrhea	-
Lactobacillus casei	NA	DNBS-induced colitis
Bacillus polyfermenticus	NA	DSS-induced colitis and TNBS-induced colitis

* Abbreviations: DNBS, dinitrobenzene sulfonic acid; DSS, dextran sodium sulfate; IL-10, interleukin 10; NA, not available; TNBS, trinitrobenzene sulfonic acid.

3) *Lactococcus lactis*

There have been many efforts to understand about LAB properties for application in the industry. *Lactococcus lactis* (*L. lactis*) is easy to manipulate and understand the genetic level of *L. lactis* (Bredmose et al., 2001). For this reason, *L. lactis* is widly used in these dayes as a protein expression factory for a high yield of the recombinant protein (Roshan et al., 2012). Recently numerous genetic tools have been developed showing that these genomes were sequenced and analyzed in this species (Bolotin et al., 2001). Many heterologous proteins such as enzymes, cytokines, allergens, antigens and reporter proteins are successfully produced in *L. lactis* (Le Loir et al., 2005).

To promote the expression of recombinant protein in *L. lactis,* some inducible and constitutive expression system have been developed by introduction of genetic regulatory cassette (Bahey et al., 2010). For example, the inducible expression system such as nisin-inducible controlled gene expression (NICE) derived from the nisABTCIPRKEFG operon is available in some *L. lactis* strains (Kuipers et al., 1998). For the special purpose, specific cassette such as signal peptide and targeting ligand have been developed (Morello et al., 2008).

2. Recombinant protein expression

1) Plasmid-based expression system

For expression of recombinant protein, plasmid-free and plasmid-based expression system are used. Plasmid-free expression system is the genetic intergration of desired gene into the host genome. For example, chemically inducible chromosomal evolution method is used to achieve multi copies of recombinant gene into the host genome (Tyo et al., 2009). But it is metabolic burden to the host and decrease the growth rate and complicate method. So plasmid-based expression system is commonly used with the development of biotechnology (Li et al., 2012).

2) Recombinant protein expression strategy

Generally, three strategies have been studied for a plasmid-based expression system.

① High copies plasmid-based expression vector system
② Construction of gene multimerization
③ Find new strong promoter

But high copies plasmid-based expression system and gene multimerization can be caused some problems, such as a metabolic load on the host, hard to replicate very large sequences of DNA, too large to be transformed and cause genetic instability (Kristala et al., 2000). Searching method of new strong promoter is direct sequencing. But it is very difficult to predict promoter strength in target host (Yang et al., 2013).

3. *Cis*-acting elements

1) Promoter

A promoter is a regulatory region of DNA located upstream of a gene, providing a control point for regulated gene transcription (Kai et al., 2011). Generally, promoter contains some special sites to initiate the transcription such as -35 and -10 hexamers called TATA box that can bind to RNA polymerase and sigma factors and RBS that can recognized by ribosomes and tRNA. Commonly, inducible and constitutive promoters have been used for the high expression of recombinant proteins (Kuipers et al., 1997). Inducible promoter can express the protein when there is an inducer. For example, there are various inducible systems in *L. lactis* which include sugar inducible expression systems, phage induced expression systems, thermal and pH induction, Nisin-controlled expression system and $P_{(Zn)}$zitR expression system (Roshan et al., 2012). Otherwise, *usp45, tuf, pfkA* and *dnaJ* promoters are also known as constitutive promoters for *L. lactis* (Kim et al., 2009). Recently, there are some strategies for the generation of the artificial promoter (Yang et al., 2013) and the modification of tandem repeated promoter (Li et al., 2012).

2) *Cis*-acting elements

A *cis*-acting element or *cis*-regulatory element regulates the gene expression on the same DNA strand and may be located in upstream or downstream of the coding region. One example of *cis*-acting elements is the *lac* operon (Carey et al., 2012). Associated with *cis*-acting elements, transcription factors have been studied. And by modification of *cis*-acting elements could lead to increase the interaction with the transcription factors (Rafael et al., 2012). The *cis*-regulatory mutations were known to have evolutionary significance (Wray, 2007).

4. The *tuf* gene

The *tuf* gene plays a role in the translation phased for an elongation of polypeptide which encodes peptide chain elongation factor Tu (EF-Tu) (Fu et al., 2012). There are three steps for the polypeptide elongation. First, EF-Tu binds GTP to the codon-dependent aminoacyl-tRNA and enter the A site of the ribosome. By GTP hydrolysis, EF-Tu-GDP is released from the ribosome and mRNA complex. Second, elongation factor Ts (EF-Ts) helps to replace EF-Tu bound GDP to GTP. Third, elongation factor G (EF-G) translocates the mRNA for the arrival of the new EF-Tu bound with GTP and aminoacyl-tRNA in the A site (Riis et al., 1990).

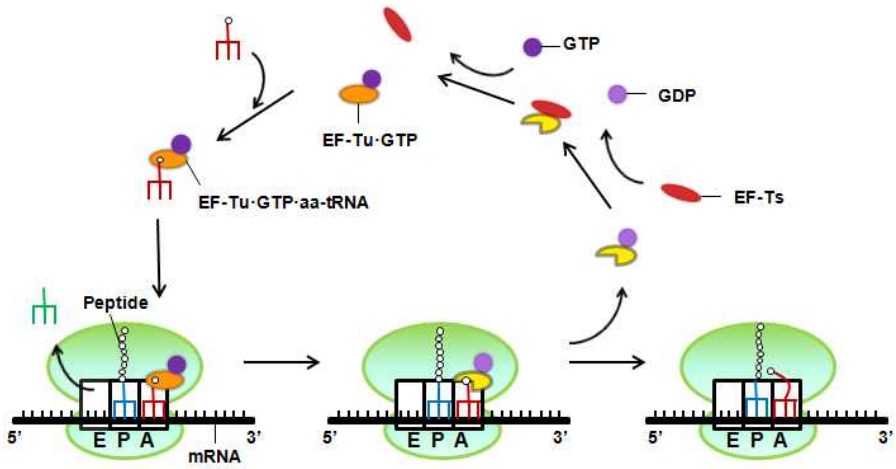


Figure 1. The elongation phase of protein synthesis. And the role of EF-Tu in this process.

III. Materials and Methods

1. Bacterial cultivation

1) Culture medium

Lactococcus lactis subsp. lactis IL1403 were cultivated in M17 broth or agar containing 0.5 % (w/v) glucose (M17G) (Terzaghi and Sandine, 1975). Erythromycin (5 ug/ml) were supplemented for transformant selection.

2) Cultivation and harvest of bacterial cells

Single colony of bacterial cells was inoculated into fresh M17G broth media and cultured for overnight at 30℃ without shaking. The resulting culture was inoculated into fresh M17G broth media and cultivated for 24h. In the indicated time point, bacterial cells were harvested by centrifugation at 5,000 g for 10 min at 4℃.

2. Transformation of bacteria

1) Preparation of IL1403 competent cells

Competent cells of wild type IL1403 for electroporation were prepared as described previously (Alegre et al., 2004). 0.5 ml of wild type IL1403 cultured for overnight was inoculated into 50 ml of fresh M17G and cultured until 0.8 of OD600 (after 3~4 h of incubation). The cells were harvested by centrifugation at 5,000 g for 10 min at 4℃. The cell pellet were washed once with distilled water, twice with 25 ml of 10 mM $MgCl_2$ and once with 20 ml of solution containing 0.5 M sucrose and 10 % (v/v) glycerol. And 1 ml of the solution of 0.5 M sucrose and 10 % (v/v) glycerol was added and cells resuspended. Finally the cells were stored at -80℃.

2) Transformation of bacterial cells

Competent cells of wild type IL1403 were mixed with plasmid DNA and transformed by electroporation using the Gene Pulser Xcell System (Bio-Rad, USA). Electroporation was performed in an electro-cuvette (1 mm gap) under the condition of 2.5 kV, 10 uF, and 300 ohm. After electroporation, the cells were resuspended with 1 ml of fresh M17G broth medium and cultivated for 2 h.

3. DNA works

1) Plasmid

pIL252-derived vectors were used for IL1403. Specially, pIL.Ptuf.Mb used as a mother plasmid in this study was from previous study (Kim et al. 2009). Plasmid DNA vectors listed in Table 3.

The pGL3-basic vector system (Promega, USA) was used for cloning the luciferase gene.

2) Preparation of plasmid DNA

Plasmid DNA from IL1403 was isolated with the Plasmid Purification Mini Kit (Nucleogen, South Korea) according to instructions of manufacturer. IL1403 cells were harvested from 5ml culture, and resuspended with 250 ul of a resuspension buffer including 100 ug/ml of RNase A, 25 % (w/v) of sucrose and 30 mg/ml of lysozyme. Then, the samples were incubated at 37℃ with shaking. After 10 min, 250 ul of a lysis buffer was added, and mixed by inverting the tube 5 times. Then 350 ul of a neutralization buffer was added and mixed by inverting 5 times. And the tube was centrifuged at 16,000 g for 10 min at 4℃ and supernatant transferred into the column and centrifuged at 16,000 g for 1 min and discarded filterate in collection tube and added 500 ul of washing buffer B and centrifuged at the same condition. After centrifugation, filterate was discarded and 750 ul of washing buffer A containing 70 % (v/v) ethanol was added and centrifuged at the same condition. The column were

centrifuged briefly to dry the filter membrane. After centrifugation, put the column into a clean 1.5 ml tube and eluted with 30 ul of distilled water and let it incubate for 1 min. Then, centrifuged at the same condition.

Table 3. Plasmids and their characteristics.

Plasmids	Relevant characteristics	Origin
pGL3-basic	amp, luciferase gene	Promega
pIL252	*ermAM*, 4.6 kb, low-copy number plasmid vector	(Simon and Chopin 1998)
pIL.CatT	pIL252-derivative,Promoterless *cat* gene, T*pepN*	(Kim et al. 2009)
pIL.Ptuf.Mb	pIL.CAtT-derivative, *tuf* promoter,*bmpB* gene	(Kim ea al. 2009)
pIL.Ptuf.Luc(X)	pIL.Ptuf.Mb-derivative, luciferase gene	This work
pIL.Ptuf.Luc(E)	pIL.Ptuf.Mb-derivative, luciferase gene with T*rrnB*	This work
pIL.Luc-t1	pIL.Ptuf.Luc(X)-derivative, modified *tuf* promoter version 1	This work
pIL.Luc-t2	pIL.Ptuf.Luc(X)-derivative, modified *tuf* promoter version 2	This work
pIL.Luc-t3	pIL.Ptuf.Luc(X)-derivative, modified *tuf* promoter version 3	This work
pIL.Luc-t4	pIL.Ptuf.Luc(X)-derivative, modified *tuf* promoter version 4	This work
pIL.Luc-t5	pIL.Ptuf.Luc(X)-derivative, modified *tuf* promoter version 5	This work
pIL.Luc-t6	pIL.Ptuf.Luc(X)-derivative, modified *tuf* promoter version 6	This work
pIL.Luc-t7	pIL.Ptuf.Luc(X)-derivative, modified *tuf* promoter version 7	This work
pIL.Luc-t2-1	pIL.Ptuf.Luc(X)-derivative, remodified t2 promoter version 2-1	This work
pIL.Luc-t2-2	pIL.Ptuf.Luc(X)-derivative, remodified t2 promoter version 2-2	This work
pIL.Luc-t2-3	pIL.Ptuf.Luc(X)-derivative, remodified t2 promoter version 2-3	This work
pIL.Luc-t4-1	pIL.Ptuf.Luc(X)-derivative, remodified t4 promoter version 4-1	This work
pIL.Luc-t4-2	pIL.Ptuf.Luc(X)-derivative, remodified t4 promoter version 4-2	This work
pIL.Luc-t4-3	pIL.Ptuf.Luc(X)-derivative, remodified t4 promoter version 4-3	This work

3) Enzyme treatment

To modify plasmid DNA used enzymes such as restriction enzymes (*Bgl*Ⅱ, *Nde*Ⅰ, *Xma*Ⅰ and *EcoR*Ⅰ), DNA dephosphorylation by CIAP and DNA ligation by T4 DNA ligase according to manufacturer's instructions. For increasing the transformation efficiency, 500~800 ng of plasmid DNA digested with restriction enzymes was used to ligate with insert DNA fragments.

4) PCR reaction

To amplify DNA , the 2x PCR master mix solution (*i-Pfu*™ or *i-Taq*™ DNA polymerase) (iNtRON, South Korea) was used according to manufacturer's instructions. For detection of insert DNA fragments, the 2x PCR master mix solution (*i-Taq*™ DNA polymerase) was used. Briefly, 10 ul or 25 ul of the 2x PCR master mix solution, 5 pmole per each primer, 1 ul of DNA template and sterile distilled water up to 20 ul or 50 ul were mixed. And PCR was performed by iCycler (Bio-RAD, USA).

5) PCR purification

All PCR products were purified by the PCR/Gel Combo Kit (Nucleogen, South Korea) for isolation the DNA from PCR mixture according to manufacturer's instructions.

6) Analysis of nucleotide sequences

Primers were from Bioneer (South Korea) and are listed in Table 4. DNA was sequenced using Applied Biosystems 3730xl (NICEM, South Korea). To validate the sequencing quality, the Chromas software was used with raw data. And BLAST (McGinnis and Madden, 2004) was searched for sequence similarity.

Table 4. Primers used in this study.

Primer Names	Sequences	Length (bp)
Luc-F	GGAATTCCATATGGAAGACGCCAAAAACAT	30
Luc-R	GGAATTCCAAATCCGCTCCCGGCGGATTTG TTACACGGCGATCTTTCC	48
Luc-R1	TCCCCCCGGGTTACACGGCGATCTTTCC	28
seqptuf-F120	GAACGGTAGTTTGCTTTATGCAG	23
seqLuc-F501	CGTCACATCTCATCTACCTC	20
seqLuc-R650	ACGCAGGCAGTTCTATGAGG	20
seqLuc-F1011	GCAAGGATATGGGCTCACTG	20
seqLuc-R1140	TCCAGATCCACAACCTTCGC	20
1-pILPtuf-F	GTCGCTATCTGTTGCGACAA	20
tuf-F	CCGGCTTCTTGAAAAAATGCGATTAAAAGC TG	32
tuf-F1	GGAAGATCTGGCTTCTTGAAAAAATGCG	28
tuf-R	GGGGAATTTGTTTTATTATAGGTAGTCTG C	30
tuf-R1	TTTTAATCGCATTTTTTCAAGAAGCCGGGG GGAATTTGTTTTATTATAG	49
tuf-R2	GGAATTCCATATGAAAAATGTCTCCTTTGG GGAATTTGTTTTATTA	46
tuf-R21	GGAATTCCATATGTTTAAAAATTTCTCCT TTGGGGAATTTGTTTTATTA	49

* under line: enzyme sequences

4. Luciferase assay

1) Growth phase-dependent luciferase expression

Single colony was inoculated into 5 ml of fresh M17G broth containing erythromycin (5 ug/ml) and cultivated overnight. And 50 ul of the resulting cultures were inoculated into 50 ml of fresh M17G broth containing erythromycin (5 ug/ml) and grown for 24 h. At appropriate time points, 2 ml of culture samples were collected.

2) Luciferase assay

To assay the luciferase activity, the Luciferase Assay System (Promega, USA) was used in this study. 20 ul of cell suspension were transferred into a white microplate (Fisher scientific, USA) for luciferase assay. The chemiluminescence was measured by a luminometer (Infinite® 200 PRO, TECAN, Switzerland).

5. Protein works

1) Protein extraction from LAB cells

In order to extract cytoplasmic protein from IL1403, the cells were harvested from 7 ml culture and washed with PBS and resuspended in 500 ul of PBS with glass beads (0.1 and 0.5 mm diameter, Sigma) by vortexing. The cells were incubated twice in bead beater (Biospec Products, USA) for 15 min each with ice at 4℃ room. Cell-debris and glass beads were removed from the cell extract by centrifugation at 14,000 g for 10 min at 4℃.

2) Quantification of proteins

The Protein Assay (Bio-Rad, USA) and 1 mg/ml BSA solution as a standard were used for quantification of the cytoplasmic proteins according to manufacturer's instructions. Quantification was calculated from the standard curves.

3) SDS-PAGE and western blot assay

4~20 % of poly-acrylamide gels (Komabiotech, South Korea) were used in this study. Each well was loaded with 40 ug of IL1403 proteins. For confirmation of molecular weight, Precision plus protein standards (Bio-Rad, USA) was used as a marker. After running SDS-PAGE, the proteins were transferred from poly-acrylamide gel to Protran nitrocellulose membranes (Whatman, UK) as described previously (Towbin et al. 1979). To detect the luciferase, polyclonal anti-luciferase goat serum

(1:1,000, Promega, USA, Cat. #G745A), rabbit anti-goat IgG HRP-linked antibody (1:10,000, R&D System, USA, Cat. #HAF017) and ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, UK) were used according to manufacturer's instructions.

4) Intensity measurement of protein bands

Band intensity of western blot assay were analyzed by ImageJ software (Abramoff et al. 2004). All lanes were normalized by the intensity of a specific (reference) lane.

6. *In vitro* characterization

1) Growth of LAB cells

Single colony was inoculated into 5 ml of fresh M17G broth containing erythromycin (5 ug/ml), and cultivated overnight 50 ul of the cultures were inoculated into 50 ml of fresh broth media. The cells were cultivated for 24 h and an aliquot of 2 mL was taken at intervals. OD_{600} values were measured by a spectrophotometer. Graphs of time-course OD_{600} values were synthesized using the GraphPad Prism software.

2) pH measurement of LAB cells

pH of culture medium was measured using a pH meter. For calibration, calibration solutions of pH 4.01, 7.00 and 10.01 were used at every measurement according to manufacturer's instructions.

IV. Results and Discussion

1. Introduction of reporter gene with terminator

1) Cloning of luciferase gene

Luciferase gene was amplified from pGL3-basic vector system (Figure 2(a)) using Luc-F and Luc-R primers. Luc-R primers were designed to include a restriction enzyme site (*EcoR* I or *Xma* I) and T*rrnB* terminator sequences (Table 4) which were elongated with the luciferase gene by touch-down PCR method for replacing the original T*pepN* terminator.

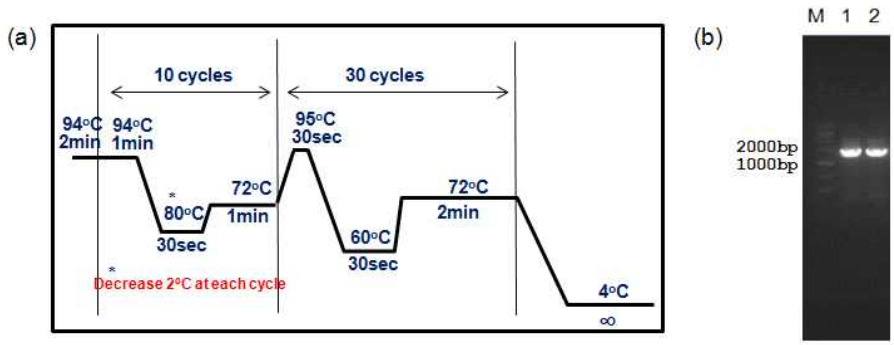


Figure 2. Amplification of the luciferase gene with T*rrnB*. (a) PCR condition for amplification of the luciferase gene with T*rrnB*. (b) Detection of amplified luciferase gene (1653 bp) with T*rrnB* (23 bp). (M: 1kb DNA ladder, 1~2: Luciferase gene with T*rrnB*)

2) Vector construction

To compare the terminator activity of T*pepN* and T*rrnB*, the luciferase gene was joined either with *tuf* promoter and T*pepN* terminator (pIL.Ptuf.Luc(X)) or *tuf* promoter and 23 bp T*rrnB* terminator (pIL.Ptuf.Luc(E)).

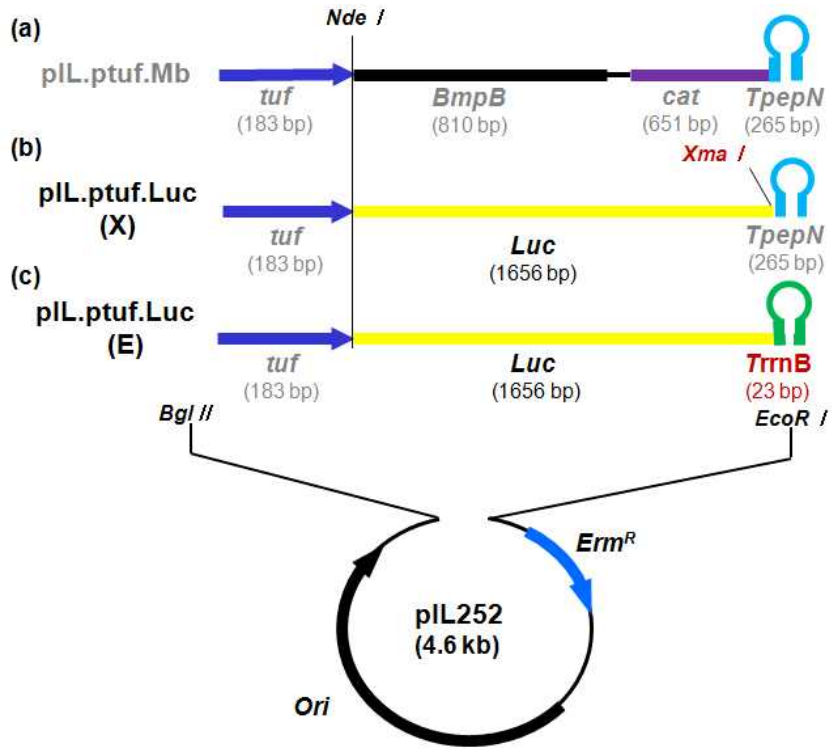


Figure 3. Schematic view of vector maps. (a) Original vector with *tuf* promoter and T*pepN* terminator (Kim et al. 2009). (b) Coding region of original vector was replaced with a luciferase gene and (c) original vector replaced with a luciferase gene and T*rrnB* terminator.

2. Validation of reporter gene and terminator

1) Growth characteristics

Two IL1403s transformed with pIL.Ptuf.Luc(E) or pIL.Ptuf.Luc(X) were cultured in M17G broth media and tested for the growth curve and mediun pH change compared with wild-type strain (no vector) as a control.

Wild-type was cultivated in plain M17G and transformants were cultivated in M17G supplemented with erythromycin. Wild-type showed slightly faster growth and resultant pH drop compared to transformants (Figure 4). Two transformants had almost same growth curve and pH drop tendency.

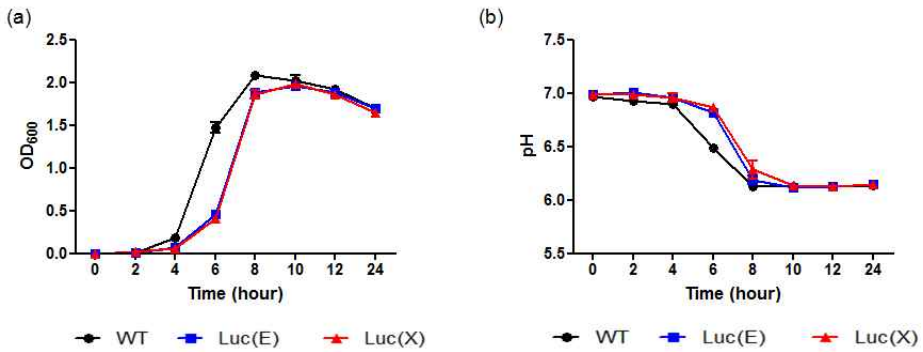


Figure 4. Growth characteristics of recombinant IL1403s. The cells were collected at 2h intervals up to 12 h period and at 24 h. (a) Growth curve and (b) pH of culture media: wild-type and transformants with Luc(E) and Luc(X) (n=3). Luc(E), pIL.Ptuf.Luc(E); Luc(X), pIL.Ptuf.Luc(X).

2) Luciferase assay

Three recombinant IL1403s were cultivated and carried out to evaluate the transcriptional terminator. After sampling, cell suspension was analyzed by luciferase assay using a luminometer. As shown in Figure 5, two vectors that had the luciferase genes showed significantly higher luciferase activity than mother plasmidas expected. The clones with T*pepN* showed higher activity compared to T*rrnB* terminator at early growth phase of 2~6 h and enzyme activity dropped quickly on and after 8 h growth. This sudden drop of enzyme activity is more dramatic when the luciferase activity normalized with cell density (Figure 5(b)).

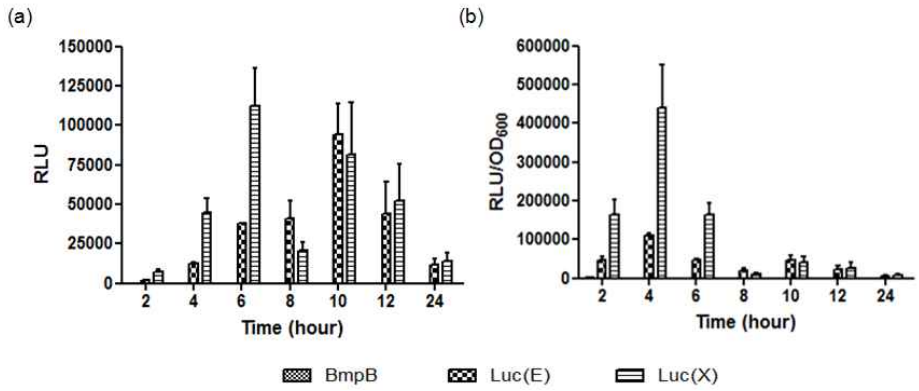


Figure 5. Luciferase activity assay. *L. lactis* IL1403 transformed with pIL.Ptuf.Mb, pIL.Ptuf.Luc(E) or pIL.Ptuf.Luc(X) were assayed for luciferase expression. (a) Luciferase activity (RLU) and (b) luciferase units normalized with cell density (RLU/OD_{600}) at different culture time (n=3). BmpB (Control), pIL.Ptuf.Mb; Luc(E), pIL.Ptuf.Luc(E); Luc(X), pIL.Ptuf.Luc(X).

3. Modification of *tuf* promoter (#1)

1) Amplification of short fragments

To amplify the core region including -35 and -10 hexamers in *tuf* promoter (Figure 6), PCR was performed with tuf-F and tuf-R primers. The primers used in this step are listed in Table 4. And the sequences of *tuf* promoter were listed in Table 5. The resulting fragments were 119 bp core *tuf* region without RBS and enzyme sites (Figure 6(a)). PCR reaction was performed with 20sec of extension time (Figure 6(b)).

Table 5. *tuf* promoter sequences.

Promoter (Length)	Sequence (5′-3′)
tuf (189 bp)	*AGATCT*ATTTTACTACTTCATAGATAAAAAAG CTAGATATATGAAAAAACTTCTTGAAAAAATG CGATTAAAAGCTGATAAGACTTGCATTTCAAA ACTATTTTAAGTATAATGATAAAGAACGGTAG TTTGCTTTATGCAGACTACCTATAATAAAACA AATTCCCCAAAGGAGACATTTTT*CATATG*

* *Green*: Enzyme sequence (*Bgl*Ⅱ and *Nde* Ⅰ)

* Red: -35 (TTGCAT) and -10 (TATAAT) hexamers

* **Purple**: RBS

* Yellow: Amplified short fragment

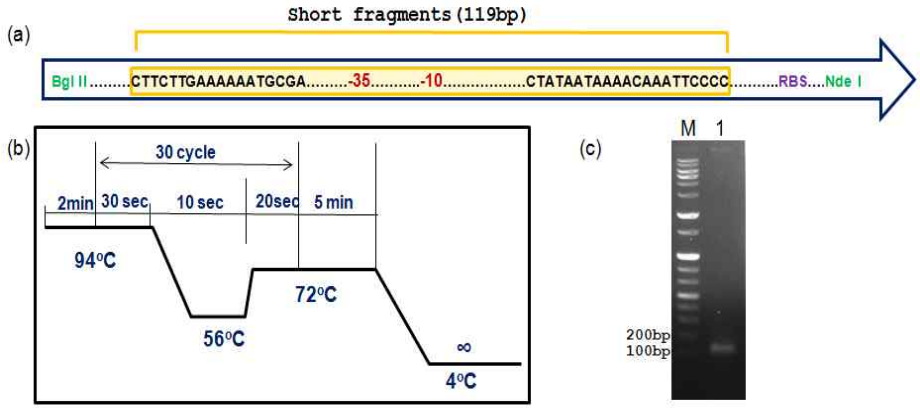


Figure 6. PCR amplification of *tuf* core region (119 bp). (a) Schematic diagram of *tuf* promoter. (b) PCR condition for amplification of *tuf* core. (c) Agarose gel electrophoresis. M: 1kb DNA ladder, 1 : *tuf* core region (119 bp).

2) Construct of repeated fragments

PCR product was used to another round of PCR to multimerize the *tuf* core using partial complementary reverse primer. This reverse primer can also bind to the 5′ region of *tuf* core at optimum annealing temperature. Optimized PCR condition was described in Figure 7(a). PCR was run with tuf-F and tuf-R1 primers at two step run conditions. PCR product was analyzed on agarose gel and showed laddering of repeated fragments (Figure 7(b)). Schematic diagram of these ladder of fragment is shown in Figure 7(c).

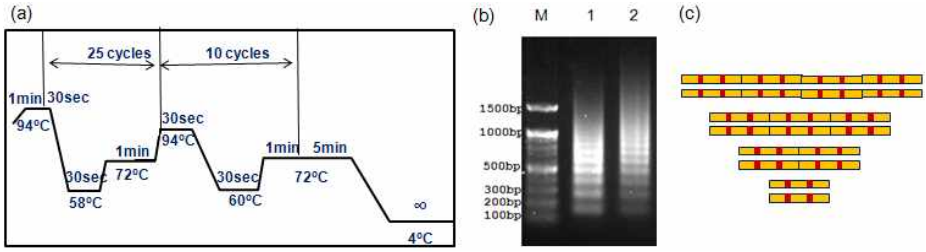


Figure 7. PCR multimerization of *tuf* core region using two step run conditions with partial complementary primer. (a) PCR conditions for construction of repeated fragments. (b) Agarose gel electrophoresis (M: 100bp DNA ladder, 1: PCR using *Tap* polymerase, 2 : PCR using *Pfu* polymerase). (c) Schematic diagram for multimerized PCR product (red: -35 and -10 hexamers, yellow: *tuf* promoter cassette).

3) Elongation of ribosome binding site (RBS) and enzyme sites

Multimerized *tuf* promoter cassettes were purified and used to template in another PCR to add, RBS and enzyme sites. For this PCR, tuf-F1 containing *Bgl*Ⅱ sequence at 5′ end and tuf-R2 including RBS sequence and *Nde* I sequence were used. The optimum condition of PCR reaction was described in Figure 8(a). PCR fragments were analyzed on agarose gel electrophoresis showing the expected DNA laddering depending on multimer number (Figure 8(b)). Final PCR products will contain *Nde* I at 5′ end, one RBS region and *Bgl*Ⅱ at 3′ end (Figure 8(c)).

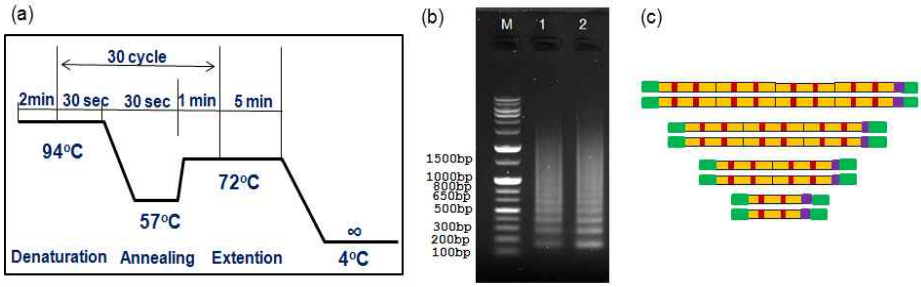


Figure 8. Construction of multimerized full cassette of *tuf* promoter repeats and RBS. (a) PCR condition. (b) Agarose gel electrophoresis (M: 1kb DNA ladder, 1: PCR using *Tap* polymerase, 2 : PCR using *Pfu* polymerase). (c) Schematic diagram of the PCR result (green: enzyme site, purple: RBS, red: -35 and -10 hexamer, yellow: *tuf* core).

4) Introduction of modified promoter (#1)

To clone the modified promoters (#1) into the vector, PCR product was purified and digested with *Bgl*Ⅱ and *Nde* Ⅰ. And pIL.Ptuf.Luc(X) was digested with *Bgl*Ⅱ and *Nde* Ⅰ and promoterless vector fragment were purified, and treated with CIAP for DNA dephosphorylation. Promoterless vector and ladders of *tuf* promoter repeats were ligated overnight at 4℃ (Figure 9). Promoters in transformants were analyzed by sequencing (Table 6).

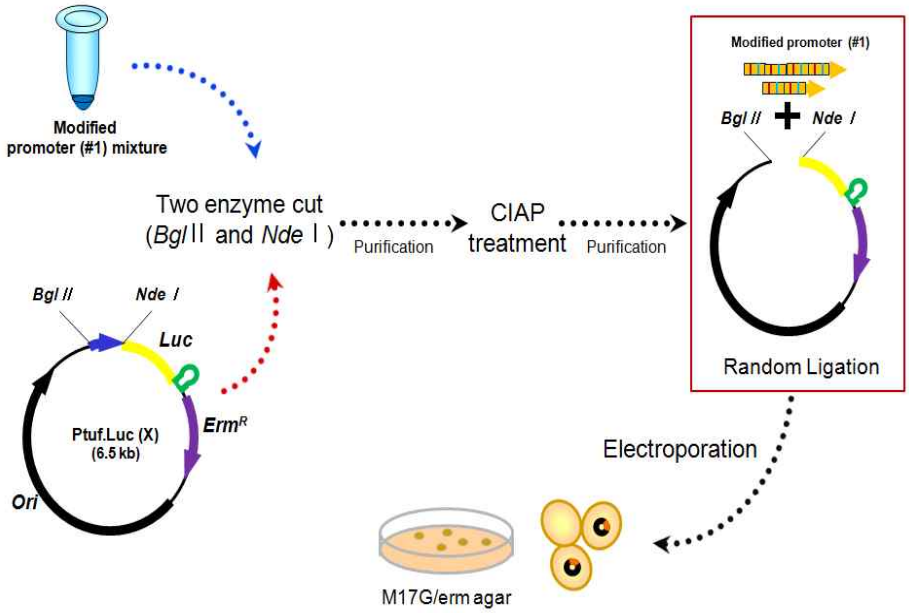


Figure 9. Schematic diagram for introduction of modified promoter (#1) into the vector. The processes are repeated to replace promoter.

Table 6. Modified promoter (#1) sequences in transformed vector.

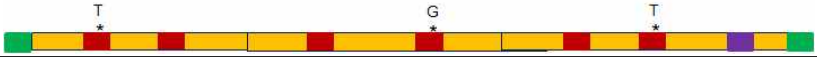
Sample name	Sequence(5′-3′)	Length
Ptuf	AGATCTATTTTACTACTTCATAGATAAAAAAGCTAGATATATGAAAA AACTTCTTGAAAAAATGCGATTAAAAGCTGATAAGACTTGCATTTCA AAACTATTTTAAGTATAATGATAAAGAACGGTAGTTTGCTTTATGCA GACTACCTATAATAAAACAAATTCCCCAAAGGAGACATTTTTCATAT G	189bp
t1	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAACTGGCCGGCTTCT TGAAAAAATGCGATTAAAAGCTGATAAGACCTGCATTTCAAAACTAT TTTAAGTATAATGATAAAGAACGGTAGTTTGCTTTATGCAGACTACC TATAATAAAACAAATTCCCCAAAGGAGACATTTTTCATATG	183bp
t2	AGATCTGGCTTCTTGAAAAAATGCGATTAAAAGCTGATAAGACTTGC ATTTCAAAACTATTTTAAGTATAATGATAAAGAACGGTAGTTTGCTT TATGCAGACTACCTATAATAAAACAAATTCCCCAAAGGAGACATTTT TCATATG	148bp
t3	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGCTGATAAGACTT GCATTTCAAAATGGAAGATCTCCGGCTTCTTGAAAAAATGCGATTAA AAACTGATAAGACTCGCATTTCAAAACTATTTTAAGTATAATGATAA AGAACGGTAGTTTGCTTTATGCAGACTATCTATAATAAAACAAATTC CCCAGGAGACATTTTTCATATG	210bp
t4	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGCTGCCATGACCTG GCAGACTACCTATAATAAAACAATTCCCCTGGCTTCTTGAAAAAATGC GTTAAAAGCTGATAAGACTTGCATTTCAAAACTATTTTAAGTATAAT GATAAAGAACGGTAGTTTGCTTTATGCAGACTACCTATAATAAAACA AATTCCCCAAAGGAGACATTTTTCATATG	219bp
t5	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAACTGATAAGACTT GCATTTCAAAAACTATTTTAAGTATAATGATAAAGAACGGTAGTTTG CTTTATGCAGACTACCTATAATAAAACAAATTCCCTCCGGCTTCTTGA AAGAATGCGATTAAAAGCTGATAAGACTTGCAGTTTGCTTTATGCAG ACTACCTATAATAAACAAATTCCCCAAAGGAGACATTTTTCATATG	235bp
t6	AGATCTCCGGCTTCTTGAAAAAATGGATTAAAAACTGATCCCAGTAC ATATAGGATTTCAAAACTATTTTAAGTATAATGATAAAGAACGGTAG TTTGCTTTATGCAGACTACCTATAATAAAACAAATTCCCTCCGGCTTC TTGAAAAAATGCGATTAAAAGCTGATAAGACTTGCATTTCAAAACTA TTTTAAGTATGATGATAAAGAACGGTAGTTTGCTTTATGCAGACCAC CTATAATAAAACAAATTCCCCCCGGCTTCTTGAAAAAATGCGATTAA AAGCTGATAAGACTTGCATTTCAAAACTATTTTAAGTTTAATGATAA AGAACGGTAGTTTGCTTTAGGCAGACTACCTATAATAAAACAAATTC CCCAAAGGAGACATTTTTCATATG	401bp
t7	AGATCTCCGGCTTCTTGAAAAAATGGATTAAAAACTGATAAGACTTG TATTTCAAAACTATTTTAAGTATAATGATAAAGAACGGTAGTTTGCT TTATGCAGACTACCTATAATAAAACAAATTCCCTCCGGCTTCTTGAAA AAATGCGATTAAAAGCTGATAAGACTTGCATTTCAAAACTATTTTAA GTATGATGATAAAGAACGGTAGTTTGCTTTATGCAGACCACCTATAA TAAAACAAATTCCCCCCGGCTTCTTGAAAAAATGCGATTAAAAGCTG ATAAGACTTGCATTTCAAAACTATTTTAAGTTTAATGATAAAGAACG GTAGTTTGCTTTAGGCAGACTACCTATAATAAAACAAATTCCCCAAA GGAGACATTTTTCATATG	395bp

* Green: Enzyme sequence (*Bgl*Ⅱ and *Nde* Ⅰ)

* Red: -35 (TTGCAT) and -10 (TATAAT) hexamers

* Orange: mutated -35 and -10 hexamers * Purple: RBS

Table 7. Schematic diagram of modified promoter (#1).

Sample name	Schematic diagram (5′-3′)
Ptuf	
t1	
t2	
t3	
t4	
t5	
t6	
t7	

* Green: Enzyme sequence (*Bgl*Ⅱ and *Nde* Ⅰ)

* Red: -35 (TTGCAT) and -10 (TATAAT) hexamers

* Purple: RBS

* Sequence change: Nucleotide sequence change are marked on top of bar.

4. Modified promoter (#1) activity assay

1) Growth characteristics (#1)

Recombinant IL1403s having various version of modified *tuf* promoter were analyzed for the growth curve and midium pH change. As shown in Figure 10, all the isolates had normal growth curve and pH drop pattern.

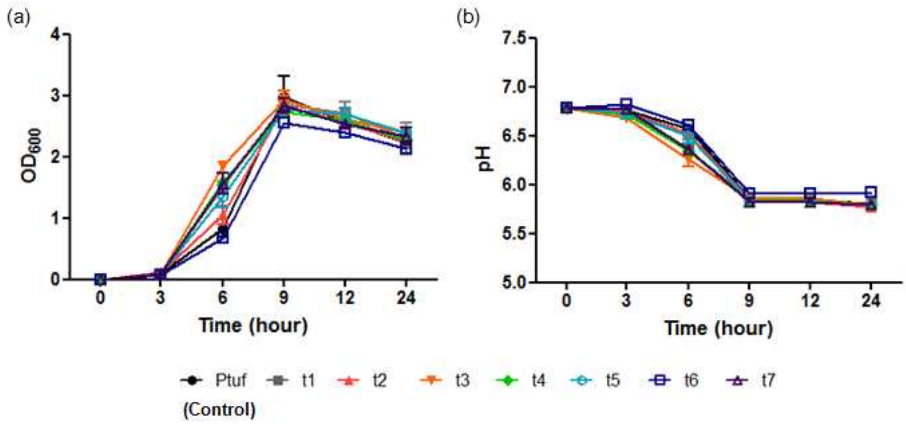


Figure 10. Growth curve of recombinant IL1403s. Cells were sampled at 3 h intervals and OD_{600} and pH was measured. (a) Growth curve and (b) pH curve of reconbinants *L. lactis* strain (n=3). Ptuf, pIL.Ptuf.Luc(X); t1, pIL.Luc-t1; t2, pIL.Luc-t2; t3, pIL.Luc-t3; t4, pIL.Luc-t4; t5, pIL.Luc-t5; t6, pIL.Luc-t6; t7, pIL.Luc-t7.

2) Luciferase assay (#1)

Luciferase was assayed at various time points of recombinant IL1403s and wild-type strain. The values of promoter strength

were measured by luminometer and shown as relative light units (RLU) (Figure 11(a) and (b)). t2, t4, t6 and t7 showed better expression compared to original promoter (Ptuf). However, the luciferase enzyme activity was not accumulated with the cell density went up. This tendency was more dramatic when activity was normalized with cell density (RLU/OD_{600}) (Figure 11(b)). As shown in growth curve (Figure 10), 3 h is the starting pint of growth having very low cell density. Thus luciferase in this configuration and strain is considered that it has very short half life, making luciferase enzyme quantification very difficult and unreliable. Nonetheless, at least initial promoter strength of t2 and t4 was much higher than control (Ptuf).

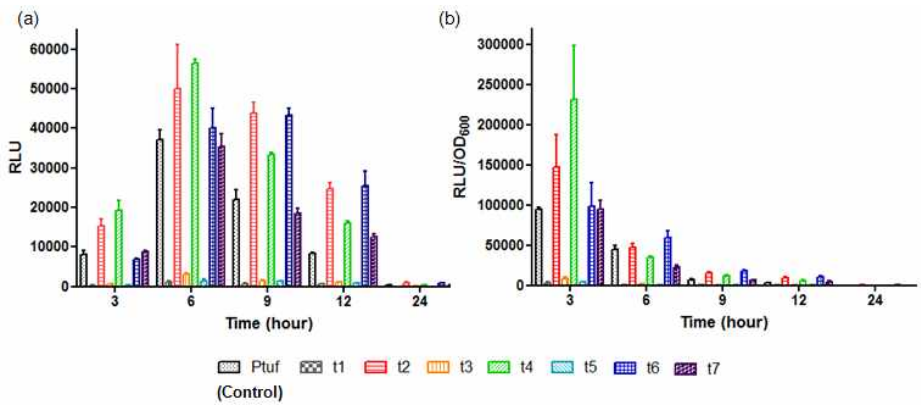


Figure 11. Luciferase activity assay. (a) Luciferase activity (RLU) and (b) luciferase units normalized with cell density (RLU/OD_{600}) of transformed IL1403s containing different promoter were assayed (n=3). Ptuf, pIL.Ptuf.Luc(X); t1, pIL.Luc-t1; t2, pIL.Luc-t2; t3, pIL.Luc-t3; t4, pIL.Luc-t4; t5, pIL.Luc-t5; t6, pIL.Luc-t6; t7, pIL.Luc-t7.

5. Construction of remodified promoter (#2)

1) Core promoter cassette preparation from modified promoter (#1)

To remodify the modified *tuf* promoters (#1), PCR was carried out with modified promoters (#1) as a DNA template and tuf-F and tuf-R primers. These primers were used to amplify the short core fragments in *tuf* promoter at previous step. But tuf-R primer can bind repeated junction in modified promoter (#1). PCR condition was same as in Figure 6(b) and PCR products suppose to show several different sizes of DNA fragments without RBS and enzyme sites.

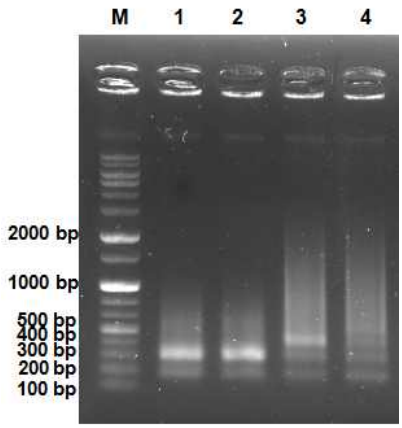


Figure 12. Amplification of core fragments from modified promoter (#1) without RBS and enzyme sites. M; 1kb DNA ladder, 1: PCR amplified from t4, 2: PCR amplified from t5, 3: PCR amplified from t6, 4: PCR amplified from t7.

2) Elongation of RBS and modified downstream sequences

The RBS downstream sequences of IL1403 genome and pIL.Ptuf.Luc(X) are slightly different. Compared with the *tuf* promoter in IL1403 genome, the *tuf* promoter in vector have inserted *Nde* I sequence at 3′ end, thus AAA(ATG) is changed to CAT(ATG) in vector (Figure 13). To restore this sequence and to change 'C' to the 'A' at downstream RBS. PCR was performed with tuf-F1 and tuf-R21 containing the enzyme sites and RBS region (Table 4). PCR condition was same as in Figure 8(a). PCR products were purified and ligated into the vector. Sequencing results were listed in Table 8. As shown in sequencing data, the sequence was restored and made the appropriate changed.

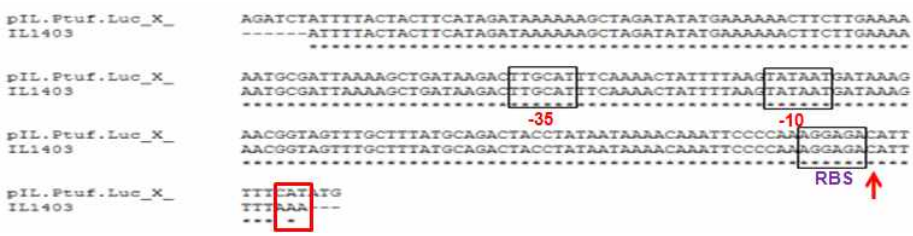


Figure 13. Alignment result of tuf promoter sequences of pIl.Ptuf.Luc(X) and IL1403 genome. ClustalW was used to compare these two sequences. Black boxes indicated -35, -10 haxamers and RBS region. Red box and arrow indicated target nucleotides that replace to 'A' nucleotide.

Table 8. Remodified promoter (#2) sequences in transformed vector.

Sample name	Sequence(5′-3′)	Length
t2-1	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGTGAA AAGACTTGCATTTCAAAACTATTTTAAGTATGATGATAA AGAACGGTAGTTTGCTTTAGGCAGACTACCTATAATAAG ACAAATTCCCCAAAGGAGAAATTTTTAAACATATG	152bp
t2-2	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGCTGA TAAGACTTGCATTTCAAAACTATTTTAAGTATGATGATA AAGAACGGTAGTTTGCTTTATGCAGACTACCTATAATAA AACAAATTCCCCAAAGAGAAATTTTTAAACATATG	152bp
t2-3	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGTGAA AAGACTTGCATTTCAAAACTATTTTAAGTTTAATGATAA AGAACGGTAGTTTGCTTTAGGCAGACTACCTATAATAAA ACAAATTCCCCAAGGAGAAATTTTTAAACATATG	151bp
t4-1	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGCTGC CATGACCTGGCAGACTACCTATAATAAAACAATTCCCCCG GCTTCTTGAAAAAATGCGATTAAAAGCTGATAAGACTTG CATTTCAAAACTATTTTAAGTATAATGATAAAGAACGGT AGTTTGCTTTATGCAGACTACCTATAATAATACAAATTC CCCAAAGGAGAAATTTTTAAACATATG	223bp
t4-2	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGCTGA TAAGACTTGCATTTCAAAACTATTTTAAGTTTAATGATA AAGAACGGTAGTTTGCTTTATGCAGACTACCTATATTAA AACAAATTCCCCCCGGCTTCTTGAAAAAATGCGATTAAA AGCTGATAAGACTTGCATTTCAAAACTATTTTAAGTATG ATGATAAAGAACGGTAGTTTGCTTTATGCAGACTACCTA TAATAAAACAAATTCCCCAAAGGAGAAATTTTTAAACAT ATG	276bp
t4-3	AGATCTCCGGCTTCTTGAAAAAATGCGATTAAAAGCTGA TAAGACTTGCATTTCAAAACTATTTTAAGTATGATGATA AAGAACGGTAGTTTGCTTTATGCAGACTACCTATAATAA AACAAATTCCCCCCGGCTTCTTGAAAAAATGCGATTAAA AGCTGATAAGACTTGCATTTCAAAACTATTTTAAGTTTA ATGATAAAGAACGGTAGTTTGCTTTAGGCAGACTACCTA TAATAAAACAAATTCCCCAAAGGAGAAATTTTTAAACAT ATG	276bp

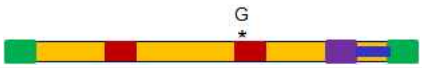
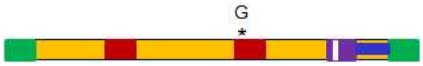
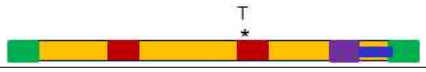
* Green: Enzyme sequence (*Bgl*Ⅱ and *Nde* Ⅰ)

* Red: -35 (TTGCAT) and -10 (TATAAT) hexamers

* Orange: mutated -35 and -10 hexamers * Purple: RBS

* under line: Modified sequence (from 'CATTTTTCAT')

Table 9. Schematic diagram of remodified promoter (#2).

Sample name	Schematic diagram (5′-3′)
t2-1	
t2-2	
t2-3	
t4-1	
t4-2	
t4-3	

* Green: Enzyme sequence (*Bgl*Ⅱ and *Nde* Ⅰ)

* Red: -35 (TTGCAT) and -10 (TATAAT) hexamers

* Purple: RBS

* Blue: Modified sequence from 'CATTTTTCAT' to 'AATTTTTAAA'

* Single nucleotide change are marked above the bar graph.

6. Remodified promoter (#2) activity assay

1) Luciferase assay (#2)

To evaluate promoter strength, cells were harvested at 6 h and 9 h of culture time. All assay procedures were same as before. As shown in Figure 14, t2-1, t4-1 and t4-2 showed high luciferase activity.

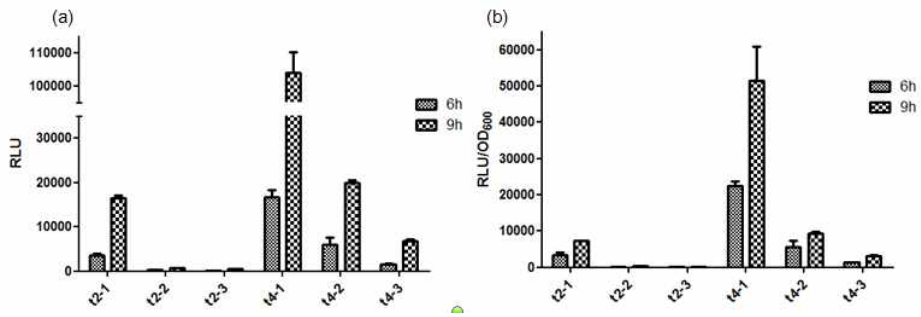


Figure 14. Luciferase assay of remodified promoter (#2) clones. (a) Luciferase activity (RLU) and (b) normalized luciferase activity (RLU/OD_{600}) (n=3). t2-1, pIL.Luc-t2-1; t2-2, pIL.Luc-t2-2; t2-3, pIL.Luc-t2-3; t4-1, pIL.Luc-t4-1; t4-2, pIL.Luc-t4-2; t4-3, pIL.Luc-t4-3.

2) Final luciferase assay

To compare the promoter strength among modified (#1) and remodified (#2) promoters, luciferase assay was carried out with original *tuf* promoter (control), selected (#1) promoters (t2, t4, t6, t7) and (#2) promoters (t2-1, t4-1, t4-3). t2, t4 and t4-1 showed higher promoter strength compared to the original *tuf* promoter (control) (Figure 15). Moreover, t4-1 showed higher reading compared to t4, indicating the modification strategy of the sequence between RBS and ATG was right (Figure 15 and Figure 16). There two vectors have same promoter, RBS, luciferase gene, terminator except the sequence between RBS and ATG. t4-1 has 'AATTTTTAAA' instead of 'CATTTTTCAT'. On the other hand, the same modification was made between t2 and t2-1. But in this case, t2-1 has lower activity compared to t2. This is opposite result. This could be explained by the unexpected sequence change in -10 region of t2-1 from 'TATAAT' to 'TATGAT'. This single nucleotide sequence change may lead a transcription efficiency drop in t2-1 compared to t2. Unfortunately, there are no clones having promoter sequence in t2, thus it is impossible at this time to conclude this explanation.

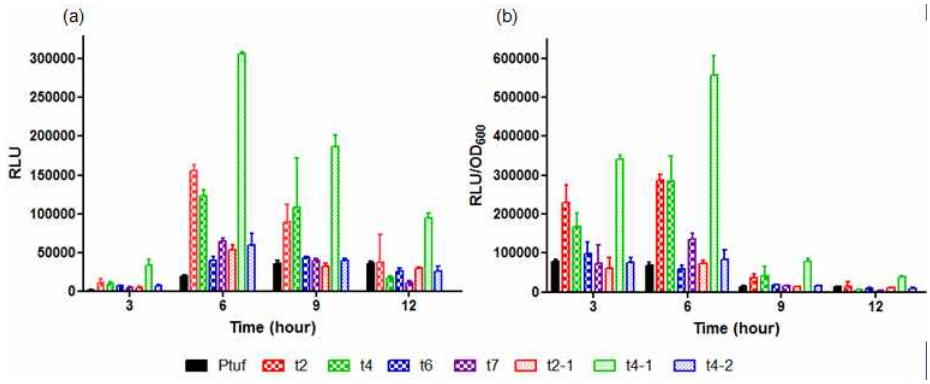


Figure 15. Luciferase assay of selected modified *tuf* clones. (a) Luciferase activity (RLU) and (b) normalized luciferase activity (RLU/OD_{600}) (n=3). Ptuf, pIL.Ptuf.Luc(X); t2, pIL.Luc-t2; t4, pIL.Luc-t4; t6, pIL.Luc-t6; t7, pIL.Luc-t7; t2-1, pIL.Luc-t2-1; t4-1, pIL.Luc-t4-1; t4-2, pIL.Luc-t4-2.

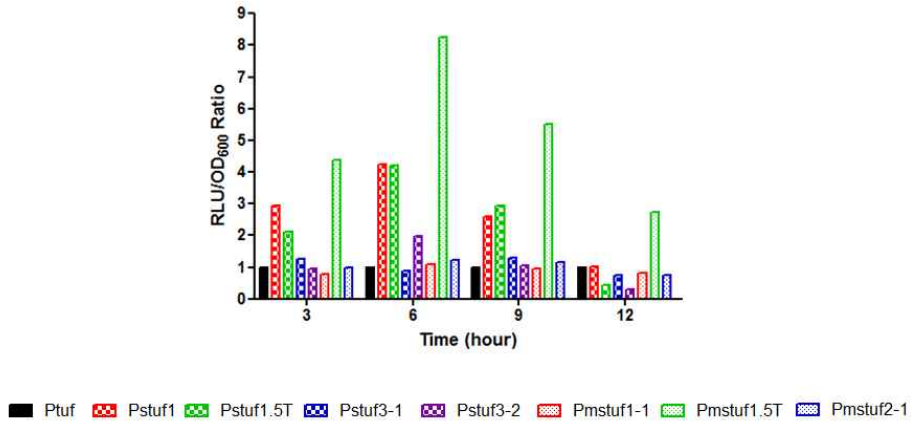


Figure 16. Normalized luciferase activity with cell density (RLU/OD_{600}) was expressed as ratio relative to control. Thus each RLU/OD_{600} was divided by control. Ptuf, pIL.Ptuf.Luc(X); t2, pIL.Luc-t2; t4, pIL.Luc-t4; t6, pIL.Luc-t6; t7, pIL.Luc-t7; t2-1, pIL.Luc-t2-1; t4-1, pIL.Luc-t4-1; t4-2, pIL.Luc-t4-2.

7. Confirmation of Protein expression

1) SDS-PAGE and western blot assay

To further check the luciferase expression among clones in protein level, SDS-PAGE and western blot were analyzed. In SDS-PAGE gel, the luciferase bands (61 kDa) was not detectable. In western blot, however, luciferase can determine the expression efficiency of recombinant protein. As shown in Figure 17(b), t4-1 showed the most strong signals. The intensity of t2, t4 and t4-1 showed stronger signal compared to control one. For cell extracts, in this study, 6 h, 8 h and 10 h culture samples were used. This time points are well from early log phase to stationary phase, thus normalized luciferase activity is very low compared to the early growth phase. Thus, the actual modified *tuf* promoter strength might be much under estimated in this study.

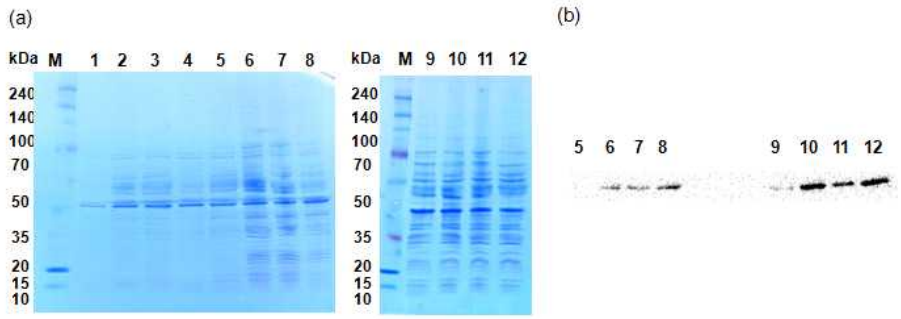


Figure 17. Confirmation of luciferase expression (61 kDa) depending on several time points. (a) SDS-PAGE and (b) western blot of cytoplasmic protein (25 ug). This figure is a representative result from Ptuf (control) and independent transformed IL1403s. M: protein marker, 1: Ptuf (6 h), 2: t2 (6 h), 3: t4 (6 h), 4: t4-1 (6 h), 5: Ptuf (8 h), 6: t2 (8 h), 7: t4 (8 h), 8: t4-1 (8 h). 9: Ptuf (10 h), 10: t2 (10 h), 11: t4 (10 h), 12: t4-1 (10 h).

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VI. Summary in Korean

여러 박테리아에서 이종 단백질의 생산율을 높이고자 하는 연구는 많이 진행 되어 왔다. 특히, 플라스미드 기반의 발현 시스템은 쉽고 유용한 수단으로 재조합 단백질 생산에 많이 이용되고 있다. 재조합 단백질의 발현율을 높이기 위해 사용되는 전략은 다음과 같이 크게 3가지 방법이 있다. 첫째로, copy 수가 높은 origin을 도입하는 것이다. 두 번째는 재조합 단백질을 코딩하는 유전자를 두 세트 이상을 붙여 벡터에 삽입하는 유전자 multimerization 방법이다. 세 번째는 강력한 프로모터를 도입하는 것이다. 하지만 이 방법들에는 숙주 내에서 벡터가 복제되기 어렵고, 도입되는 유전자가 너무 커 숙주 내로 형질전환 되기 유전적으로 불안정하고, 프로모터의 효율을 예측하기가 어렵다는 한계점들이 있다. 이에 본 연구에서는 이미 잘 알려져 있는 프로모터를 변형시키고 그들의 프로모터 효율을 테스트하였다. 또한 전사 terminator와 RBS (ribosome binding site) 와 같은 다른 *cis*-acting element를 도입하여 재조합 단백질의 발현 효율을 증진시키는 전략으로 연구를 수행하였다.

유산균 (*Lactococcus lactis subsp. lactis* IL1403)은 식품 및 축산 분야에서 널리 이용되고 있으며, 점막 면역 반응을 유도할 살아있는 경구용 백신으로도 많이 연구되어지고 있다. 그리고 유전체 내에 존재하는 translational elongation factor Tu (*tuf*) 유전자는 house-keeping gene으로, 이 유전자의 프로모터는 이미 IL1403에서 잘 작동한다고 알려져 있다. 본 연구에서는 *tuf* 프로모터를 변형시켜 리포터로써 도입된 luciferase 유전자의 발현 효율을 비교하였다.

우선 두 개의 terminator, T*rrB* (23 bp) 와 T*pepN* (265 bp)을 도입하여 이들에 의해 발현되는 luciferase의 발현 효율을 비교하였다. T*pepN* terminator가 luciferase 발현에 있어 조금 더 좋은 것을 확인 할 수 있었다.

이에 T*pepN* terminator가 도입된 플라스미드를 지속적으로 이용하여 연구를 진행하였다.

다음으로, *tuf* 프로모터의 변형의 연속과정을 시도하였다. 박테리아 내에는 전사 개시 부위 상단 -35와 -10 부분이 있어 전사 개시에 필요한 RNA polymerases나 여러 sigma factor들이 인지하고, 모인다. 이러한 *tuf* 프로모터 내의 -35와 -10 부분을 포함하는 119 bp의 core 부분을 증폭하고, 일부 상호보완적인 부분을 갖는 reverse primer와 함께 일련의 PCR기법을 수행하여 변형시킨 *tuf* 프로모터를 구축하였다. 이렇게 구축된 PCR 결과물 (#1)을 프로모터 부분을 제거한 pIL.Ptuf.Luc(X) 벡터에 도입하였다. 결과물 중 t2, t4, t6 과 t7이 control인 *tuf* 프로모터보다 더 높은 luciferase 활성을 갖는 것을 확인 할 수 있었다. 특히나 t2 와 t4 가 더 효율이 좋았다. 이에 이 두 프로모터를 다음 실험에 이용하였다.

RBS와 개시 코돈 (ATG) 사이의 서열이 단백질 번역과정의 효율에 있어 중요하다는 것은 잘 알려져 있다. 이에 이 부분의 서열을 'CATTTTTCAT'에서 'AATTTTTAAA'로 AT-rich하게 변형시켜 주었다. 이 변형 과정은 이미 선별된 프로모터에서 수행하여, 새로운 시리즈의 *tuf* 프로모터 cassette (#2)를 구축하였다. 변형된 프로모터 (#2)가 포함된 transformed IL1403s으로 luciferase 활성을 비교하였다. t2와 t4에서 유래된 t2-1과 t4-1의 luciferase 활성이 더 높은 것을 확인 할 수 있었다.

모든 변형된 *tuf* 프로모터를 갖는 clone들의 luciferase 활성을 비교한 결과, t4-1가 기존의 t4 보다 훨씬 높은 수준의 활성을 갖는 것을 확인 할 수 있었다. 이는 RBS와 개시코돈 사이의 서열이 단백질 발현에 있어 중요하다는 것을 나타낸다. 이 결과를 조금 더 확인하기 위해, luciferase의 발현을 SDS-PAGE와 western blot을 통해 단백질 수준에서 분석하였다. SDS-PAGE에서는 61 kDa의 luciferase를 확인 할 수 없었지만, western blot에서는 t2, t4, t4-1를 갖고 있는 clone들이 기존의 tuf 프로모터를 갖는 clone보다 더 강한 siganl

을 보였다.

결과적으로, 이 연구를 통해 변형시켜 더 효율이 좋아진 프로모터와 추가적인 *cis*-acting elements를 도입함으로써 IL1403에서 단백질의 발현을 증진 시킬 수 있다는 것을 밝혔다. pIL252 벡터는 낮은 copy 수를 갖고 있는 플라스미드이다. 그렇기에 재조합 단백질의 발현을 증가시키기 위해, 높은 copy 수의 플라스미드로의 도입이 필요하다.

주요어: 유산균, *cis*-acting element, 프로모터, *tuf*, luciferase, 변형

VII. Acknowledgement

어느덧 '눈 깜짝할 사이에' 라는 표현처럼 2년 동안의 석사과정을 무사히 마무리하게 되었습니다. 부족한 점이 많은 저를 받아주신 최윤재 교수님께는 석사 과정 동안은 물론 지금도 그리고 앞으로도 너무 감사하다는 인사를 드리고 싶습니다. 길지도, 짧지도 않은 석사과정 동안 제가 이곳에서 얻은 것들이 너무나 많습니다. 교수님께서 주신 이 귀중한 경험, 지식 그리고 교훈들은 앞으로, 제가 박사과정을 수행하면서는 물론 살아가는데 있어 무척이나 큰 도움이 될 것 같습니다. 다시 한 번 이렇게 좋은 기회를 주셔서 정말 감사하다는 인사 이 글을 통해 전해 드립니다.

언제나 세미나 시간에 아끼지 않은 조언과 가르침을 주신 조종수 교수님, 교수님의 해박한 지식과 열정을 보며 학업의 길을 걷는 학생으로서 본받아야 한다고 늘 생각했습니다. 교수님께서 보여주신 학구적인 자세와 조언들 잊지 않고 열심히 노력하는 학생이 되겠습니다.

제 학위 심사를 맡아주신 백명기 교수님, 강상기 교수님께도 감사하다는 인사드리고 싶습니다. 학위 심사 위원장으로, 제 학위과정을 마무리 하는데 있어 많은 조언 주신 백명기 교수님 감사드립니다. 또한 제가 학위 주제를 정하고 수행하는데 있어 세심한 지도를 해주신 강상기 교수님, 너무 감사드립니다. 대학원 생활하는데 있어 교수님께서 해주신 조언들은 제게 큰 도움과 격려가 되었습니다. 앞으로도 교수님의 많은 조언과 가르침 부탁드립니다.

미생물을 전공하시고 실험실의 역사를 다 꾀고 계신 복진덕 박사님, 박사님의 코멘트들은 제 학위 주제를 구성하는데 있어 큰 도움이 되었습니다. 또한 박사님의 논리적인 사고는 과학자로서 본받아야 할 점이라고 생각합니다.

이번에 강원대학교의 교수가 되신 김은배 교수님, 교수님께서 실험실에 남겨주신 귀중한 자원들 덕분에 제 학위주제가 탄생 한 것이

아닌가 싶습니다. 잘 정리되어 있는 교수님의 자료들과 sample들은 저에게 있어 바이블이라 느껴졌습니다. 그리고 유산균 실험에 있어 경험에서 얻은 지식들을 토대로 저에게 해주신 조언들은 너무나 큰 도움이 되었습니다. 정말 감사드립니다. 그리고 앞으로도 많은 조언 부탁드립니다.

짧은 시간이지만, 논문 작성에 있어 조언을 주신 문현석 박사님 감사합니다. 박사님께서 해주신 학술적인 조언들 잊지 않겠습니다. 또한 구수한 사투리가 매력적이신 이윤석 박사님, 박사님께서 강의해 주신 세미나는 축산 분야에 대한 지식이 적은 저에게 많은 공부가 되었습니다. 감사합니다.

신사적이시고 언제나 후배들을 잘 챙겨주시는 대천오빠, 바쁘신 와중에도 틈틈이 관심 갖고 실험이 잘 진행되고 있는지 물어봐 주시고 어려움이 있을 땐 같이 고민해 주셔서 감사해요. 그리고 1층 실험실에서 열심히 연구하시는 장도 오빠, 많은 대화를 나눠 본건 아니지만 항상 노력하시는 모습이 기억에 많이 남아있네요. 오빠들 모두 한국에 오셔서 열심히 노력하신 만큼 좋은 성과를 이루시길 바래요.

귀엽고 인정 많은 혜선이~, 나보다 어린데도 혜선이한테 내가 많이 의지했던 것 같아. 대학원 입학하고 나서 실험은 물론 이것저것 내가 도움을 많이 받았어... 정말 고마워. 그리고 진지하게 연구에 임하는 모습, 멋있다고 생각해. 앞으로도 우리 잘 지내자^^. 그리고 다양한 분야에 있어 해박한 지식을 갖고 있는 창윤오빠, 낯가림이 심해 잘 어울리지 못했던 신입생 때 세심하게 배려해 주시던 모습들이 많이 생각나네요. 장난도 많이 치고 했는데 다 받아주시고... 감사해요. 책임감 강하시고 무엇이든 똑 부러지게 해내시는 준영오빠, 처음엔 무서운 선배로만 느껴졌는데 시간이 지날수록 섬세하시고 친절하다는 걸 알게 되었어요. 앞으로도 잘 부탁드려요. 그리고 똑똑하고 착한 태은언니, 처음 이 실험실을 접했을 때 친절하게 대해 주시던 언니 모습이 많이 생각나네요. 맡으신 일이 많은데도 내색

없이 자신의 연구까지 잘 해내시고 후배들 연구까지 챙기시는 모습, 정말 멋있어요. 밝고 생각이 깊은 수나, 사람들과 대화 할 땐 분위기 메이커로 실장 일을 할 때는 진지하게 최선을 다하는 모습 멋있어~. 지금은 힘들어도 수나가 노력한 만큼 나중에 보상 받을 거야. 힘내~^^. 그리고 겉은 퉁명스럽지만 사실은 굉장히 친절한 건구오빠, 힘들다고 투정부리고 장난쳐도 다 받아주시고 고민거리나 답답할 때 제 얘기 많이 들어주셔서 정말 감사해요. 오빠 덕분에 위로도 많이 받고 실험실 생활도 잘 적응할 수 있었던 것 같아요. 앞으로도 잘 부탁드려요. 그리고 똑똑하고 열정적인 원석오빠, 오빠는 졸업하시고 나서 무엇을 하시든 잘 하실 거에요. 연구에 최선을 다하시는 모습이 인상적이셨어요. 가끔 놀러오세요.

유쾌하고 활발하시지만 걱정이 많았던 성현오빠, 입학하고 나서 의지를 많이 했는데, 어느덧 저희도 졸업이네요. 2013년 이후 자주 뵙진 못했지만 밝아지신 모습이 굉장히 보기 좋네요. 오빠가 그 동안 고생하셨던 만큼 졸업 진심으로 축하드리고요. 졸업 후에도 자주 학교에 놀러와 주세요^^. 그리고 항상 밝고 쾌활한 정인이, 하나뿐인 여자 동기인 만큼 나도 모르게 정인이한테 많이 의지 했었던 것 같아. 정인이가 워낙 똑 부러지고 센스쟁이라 졸업하고 무엇을 하든 원하는 대로 잘 해낼거라 믿어 의심치 않아. 정인이도 졸업 축하해^^. 똑똑하고 배려심 많은 동석이, '인누'라고 부르면서 먼저 친근하게 말도 걸어주고 애교도 부리고 이것저것 많이 챙겨줘서 고마워. 정말 정이 많은 친구라 옆에 있음 매우 듬직해. 앞으로도 잘 부탁해~. 마지막으로 힘든 시기 같이 보낸 우리 동기들, 2년 동안 함께 해줘서.. 우정사진 찍으면서 좋은 추억 남겨줘서 모두들 정말 고마워요.

운동 좋아하고 제 연구에 무척 열심히인 호빈이, 동갑내기 친구인데 잘 챙겨주지 못 한 것 같아 미안하네ㅜ 무뚝뚝한 것처럼 보였지만 무척이나 섬세하고 잘 챙겨주는 모습에 나름 놀랐어. 열심히 노력하는 만큼 좋은 성과가 있을 거야, 힘내. 그리고 유쾌하고 매력

넘치는 윤정이, 사교적인 성격으로 선배들과도 잘 어울리고 맡은 일에 똑 부러지게 해결하는 모습이 정말 보기 좋다. 윤정이도 힘내고, 앞으로도 오래 봤으면 좋겠어~. 마지막으로 아직은 어색한 신도운씨 그리고 귀여운 신입생 이기준, 김나영, 김휘수, 윤소연. 모두 새로 시작하는 만큼 선배들에게 많이 묻고 열심히 공부하면 좋은 성과를 얻을 수 있을 거에요. 그리고 교수님의 비서로 일하고 있는 귀여운 정민이, 애교 많고 책임감 있고 성실하게 일을 수행하는 모습 보기 좋다. 정민이도 오래 봤으면 해~, 앞으로도 잘 부탁해.

대학 동기이자 내 마음 잘 알아주는 경민이, 대학교 4년 동안 붙어 다니면서 많은 추억거리 만들었는데 이렇게 대학원에 와서도 함께 하는 구나. 힘들 때마다 만나서 얘기 들어주고 위로해주고 너무 고마워. 경민이도 꼭 좋은 결과로 멋지게 졸업할 수 있을 거야. 내가 항상 응원하고 있는거 알지?! 그리고 올곧고 듬직한 선미, 지금 다니는 학교도 다르고 분야가 다르지만 최선을 다하는 선미 모습 정말 멋지다고 생각해. 선미는 꼭 좋은 선생님이 될 거야. 지금은 우리가 다 힘든 시기를 격고 있지만 선미라면 이기고 잘 해 낼거라 믿어! 힘내~. 분위기 메이커이자 마음 여린 우리 선영이, 여린 심성을 갖고 있지만 언제나 밝고 강하게 지내는 모습이 정말 멋져. 선영이도 이 힘든 시기 무척 잘 이기고 성공할거라 믿어. 파이팅! 옆 건물에 있지만 요즘 보기 힘든 소영이, 처음 대학원에 왔을 때 소영이 밖에 아는 사람이 없어서 많이 의지했는데 요즘 보기 힘드네ㅠ 연구하는데 있어 최선을 다하는 소영이 모습이 항상 인상 깊게 생각해. 박사과정을 보내면서 서로 응원해주고 힘들면 위로도 해주고 잘 지내자. 그리고 회사 생활 하면서 바쁜 시간 보내고 있는 성희, 성희도 항상 자신이 하는 일에 있어 열심히 최선을 다하는 모습이 멋지다 생각해. 얼굴 잊겠어ㅠ 빠른 시일 내에 보자!

그리고 고등학교 졸업하고 나서 더 각별해진 윤미, 수정이, 이선이. 아직 사회 초짜이고 부족한 점이 많은 날 항상 배려해주고 챙겨줘서 정말 고마워. 앞으로도 더 친하게 지내자. 가장 오래된 베프이자

몸이 약해 많이 걱정되는 은정이, 중학교 때부터 이루고자 했던 꿈을 이루고 열심히 하는 진아, 고등학교 내내 함께 하고 솔직함이 매력적인 영미. 모두들 함께해줘서 너무 고마워요.

그 누구보다 가장 감사고 존경하고 사랑하는 우리 엄마, 아빠. 본인들 보다 자식을 먼저 생각하시는 모습, 언제나 마음 저리게 느끼고 감사하고 있습니다. 제가 선택한 일에 언제든 응원해주시고 격려해주셔서 불안함 속에서도 힘을 내서 열심히 할 수 있고 앞으로도 더욱더 열심히 할게요. 평소 무뚝뚝한 첫째로 표현을 솔직히 잘 하지 못하지만, 말로 다 표현 할 수 없을 만큼 감사하고 사랑해요. 그리고 부족함이 많은 언니(누나)를 둬 고생 많은 우리 똑순이 인애, 귀여운 승훈이. 나이 들면서 점점 더 공감대도 생기고 의지도 많이 하게 되는 것 같아. 앞으로도 서로를 더 배려하고 챙기면서 돈독하게 지내자. 사랑해.

2014년 1월 25일, 관악에서

김 인 선