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Isolation and Characterization of Some Promoter Sequences from *Leuconostoc mesenteroides* SY2 Isolated from Kimchi

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Some promoters were isolated and characterized from the genome of *Leuconostoc mesenteroides* SY2, an isolate from kimchi, a Korean traditional fermented vegetable. Chromosomal DNA of *L. mesenteroides* SY2 was digested with Sau3AI and ligated with BamHI-cut pBV5030, a promoter screening vector containing a promoterless *cat-86*. Among *E. coli* transformants (TFs) resistant against Cm (chloramphenicol), 17 were able to grow in the presence of 1,000 μg/ml Cm and their inserts were sequenced. Transcription start sites were examined for three putative promoters (P04C, P25C, and P33C) by primer extension. Four putative promoters were inserted upstream of a promoterless α-amylase reporter gene in pJY15α. α-Amylase activities of *E. coli* TFs containing pJY15α (control, no promoter), pJY03α (pJY15α with P03C), pJY04α (with P04C), pJY25α (with P25C), and pJY33α (with P33C) were 66.9, 78.7, 122.1, 70.8, and 99.3 U, respectively. Cells harboring pJY04α showed 1.8 times higher activity than the control. Some promoters characterized in this study might be useful for construction of food-grade expression vectors for *Leuconostoc* sp. and related lactic acid bacteria.

Keywords: *Leuconostoc mesenteroides*, promoter screening, expression vector, chloramphenicol, α-amylase

Introduction

Lactic acid bacteria (LAB) are a group of gram-positive, non-motile, and non-spore-forming bacteria with fermentative metabolisms and low G+C% contents. LAB include many industrially important species and have been used as starters for various fermented foods such as cheese, yogurt, sauerkraut, pickle, and kimchi (a Korean traditional fermented vegetable) [1, 2]. Many species of LAB are utilized as probiotics for humans and animals too. Recently, LAB are employed as production hosts for many products, including enzymes, vitamins, metabolites responsible for flavor and taste of fermented foods, and antigens for antibody production [3, 4]. The popularity of LAB as cell factories is on the rise because of advantages of LAB. First, they are generally recognized as safe organisms, and LAB and their metabolites can be used as starters or additives for fermented foods. Second, LAB have relatively simple metabolic pathways that can be easily modified for optimum production of a specific metabolite [5].

Overexpression of a target gene is often the method of choice when overproduction of a metabolite is needed. For this, efficient expression systems are required. An expression system consists of a host cell and an expression vector. A strong and regulatable promoter is often employed in an expression vector and starts transcription of a target gene placed downstream of the promoter. Strong promoters are essential parts for expression vectors. Food-grade host cells and vectors are required when production of metabolites is intended to occur during food fermentations. All components of a food-grade vector should be originated from food-grade organisms such as LAB [6]. Not only promoters but also selection markers and plasmid vector frames, including origins for replication, should be derived from safe organisms.

Sequences from pathogens or potential pathogens such as *E. coli* cannot be used. Use of LAB as cell factories is expected to increase in the future for production of commercially important metabolites. In this respect, gene expression and regulation in LAB must be well understood, and efficient food-grade expression vectors should be made available.

Leuconostoc mesenteroides SY2 was isolated from kimchi and harbors three cryptic plasmids. pFML1 was the smallest one (4.6 kb), and an *Escherichia coli-Leuconostoc* shuttle vector, pSJE (6.6 kb, Em^r), was constructed based on pFML1 [7]. In this study, a total of 17 fragments containing putative promoters were isolated from the chromosome of *L. mesenteroides* SY2 by using a promoter screening vector, pBV5030, and sequenced [8]. Some promoters were further characterized and evaluated for their efficacies to express an α-amylase gene from *Lactobacillus amylovorus* B4540 [9]. The results indicated that some promoters can be useful for the construction of expression vectors for LAB.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this work are listed in Table 1. *L. mesenteroides* SY2 was grown in MRS (Difco Lab,

Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Description	Source or reference
E. coli		
DH5α	φ80d*lacZ⊿*M15,*recA1,endA1,gyrA96,thi-1,hdR17*(r_k^-,m_k^+), *supE44* ,*relAl, deoR, ⊿*(*lacZYA-argF*) U169	Gibco BRL
Leuconostoc mesenteroides		
SY2	Wild-type strain isolated from kimchi	[7]
Lactobacillus amylovorus B4540	Amylolytic strain	[9]
plasmid		
pBV5030	Promoter probe vector containing a promoterless *cat*-86 gene	[8]
pSJE	Shuttle vector for *E. coli* and *Leuconostoc* sp., 6,576 bp, Em^r	[7]
pSJETα	pSJE containing *amyL* as a 3.0 kb *NotI* fragment from *Bacillus licheniformis*	[13]
pCW4Tα	pCW4 containing *amyL* as a 3.0 kb *NotI* fragment from *Bacillus licheniformis*	[18]
pJY15α	pSJE containing promoterless α-amylase gene from *L. amylovorus* B4540	This study
pJY03C	pBV5030 derivative containing clone 03C, 283 bp	This study
pJY04C	pBV5030 derivative containing clone 04C, 270 bp	This study
pJY05C	pBV5030 derivative containing clone 05C, 313 bp	This study
pJY06C	pBV5030 derivative containing clone 06C, 369 bp	This study
pJY07C	pBV5030 derivative containing clone 07C, 379 bp	This study
pJY08C	pBV5030 derivative containing clone 08C, 358 bp	This study
pJY09C	pBV5030 derivative containing clone 09C, 415 bp	This study
pJY10C	pBV5030 derivative containing clone 10C, 458 bp	This study
pJY11C	pBV5030 derivative containing clone 11C, 453 bp	This study
pJY13C	pBV5030 derivative containing clone 13C, 436 bp	This study
pJY15C	pBV5030 derivative containing clone 15C, 532 bp	This study
pJY18C	pBV5030 derivative containing clone 18C, 603 bp	This study
pJY20C	pBV5030 derivative containing clone 20C, 629 bp	This study
pJY21C	pBV5030 derivative containing clone 21C, 664 bp	This study
pJY22C	pBV5030 derivative containing clone 22C, 406 bp	This study
pJY25C	pBV5030 derivative containing clone 25C, 527 bp	This study
pJY33C	pBV5030 derivative containing clone 33C, 631 bp	This study
pJY03α	pJY15α derivative containing 03C promoter	This study
pJY04α	pJY15α derivative containing 04C promoter	This study
pJY25α	pJY15α derivative containing 25C promoter	This study
pJY33α	pJY15α derivative containing 33C promoter	This study

USA) broth or agar plates (1.5% (w/v)) at 30°C. *E. coli* was grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation. Antibiotics were used at the following concentrations: erythromycin (Em), 200 µg/ml; chloramphenicol (Cm), from 20 to 1,250 µg/ml.

Screening of Promoters from *L. mesenteroides* SY2 Chromosome

Chromosomal DNA from *L. mesenteroides* SY2 was prepared according to the method of Luchansky *et al*. [10]. Twenty micrograms of chromosomal DNA was digested with Sau3AI and the fragments were cloned into the unique BamHI site of pBV5030, which contained a promoterless *cat*-86, a Cm resistance gene [8]. *E. coli* was transformed with the ligation mixture by electroporation [11]. The ligation mixture was put into a cold electroporation cuvette (0.1 cm), and a single pulse was applied (25 µF capacitance, 600 Ω resistance and a field strength of 18 kV/cm) with GenePulser II (BioRad, USA). The mixture was immediately diluted with 1 ml of LB broth, incubated for 1 h at 37°C, and then spread on LB plates with Em (200 µg/ml). *E. coli* transformants (TFs) were selected on LB agar plates containing Cm and Em (200 µg/ml). Plasmid DNAs from *E. coli* TFs were prepared by using a QIAprep kit (Qiagen, USA). Agarose gel (1% (w/v)) electrophoresis was conducted with Tris-Acetate-EDTA buffer (pH 8.0) to check the size of the plasmids. Restriction enzymes, alkaline phosphatase (Promega, USA), and T4 DNA ligase (Takara, Japan) were used according to the provided instructions.

Determination of Promoter Sequences

Primers corresponding to upstream (5′-AATTCGAGCTCGGTA CCCGG-3′) and downstream (5′-TGCAGGTCGACTCTAGAGAT-3′) of the insertion site on pBV5030 were used for sequencing of the cloned fragments. The DNA sequences were determined at Cosmogenetech (Korea). The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find homologous sequences in the GenBank database. Promoter sequences were predicted using Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). DNA structure prediction was performed using the mfold program (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form).

Transcription Start Site Analysis

Total RNA was extracted from *E. coli* TFs harboring pJY04C, pJY25C, or pJY33C. Cells were grown in LB broth with Em until the absorbance at 600 nm reached 0.5. Cells were recovered by centrifugation (7,000 ×*g*, 4°C, 20 min) and disrupted by using a Mini-Beadbeater-8 Cell Disrupter (BioSpec, USA). The FastRNA Pro Blue Kit (Qbiogene, Canada) was used to isolate total cellular RNA, and the RNA concentration was determined by a spectrophotometric method. Three oligonucleotides were used for primer extension: P04C (5′-GGTCGACTCTAGAGGATCAACTAC-3′), P25C (5′-GCGCTTTTAGTTCCTTACATACC-3′), and P33C (5′-GTAGCTATCAAGGATTGCCGTCC-3′). Primers were 5′ end labeled with [γ-^{32}P]dATP (3,000 Ci/mmol) using T4 DNA polynucleotide kinase (Promega). Each labeled primer (10 pmol) was hybridized with 0.5 µg of total RNA for 20 min at 58°C and extended with AMV reverse transcriptase (Promega) for 30 min at 42°C. The extended products were mixed with loading buffer, denatured at 90°C, and subjected to electrophoresis on a 6% polyacrylamide–8 M urea sequencing gel. As a size ladder, a DNA sequencing reaction was run in parallel, using single-stranded M13mp18 DNA and -40 universal primer (5′-GTTTTCCCAGTC ACGAC-3). For sequencing, [α-^{32}P]dATP (1,000 Ci/mmol), and Sequenase ver. 2.0 DNA polymerase (USB, USA) were used. The autoradiogram was obtained using a Fuji medical X-ray film (Fuji Film, Japan).

Construction of a Reporter Vector with a Promoterless α-Amylase Gene

A plasmid vector containing a promoterless α-amylase gene was constructed to evaluate the efficacies of selected promoters (Fig. 1). An α-amylase gene without its own promoter was amplified from an α-amylase gene of *L. amylovorus* B4540 [12]. A primer pair was used: amylF (5′-GGCTATCGATGGGGCAGT AAAAAGTG-3′, ClaI site underlined) and amylR (5′-GGCTAT CGATACTACCCCAACTTGAAGG-3′, ClaI site underlined). The amplification conditions were as follows: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. The amplified fragment was digested with ClaI and ligated with pSJE,

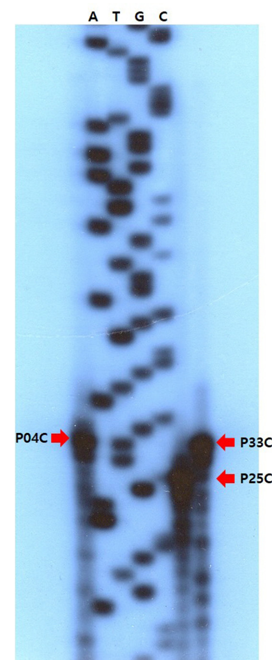

Fig. 1. Primer extension results for mRNAs prepared from *E. coli* transformants.

Primers specific for 04C, 05C, and 33C promoters were used. Mapping was done by the primer extension method with [γ-^{32}P]ATP as the label. The reaction mixtures were run on a 6% sequencing gel.

a shuttle vector for *E. coli* and *Leuconostoc* spp. [7], resulting in pJY15α (Table 1). Selected promoters were amplified by PCR using primer pairs that contained a KpnI site (forward primer) or a SalI site (reverse primer). The PCR products were inserted into pJY15α at the KpnI and SalI sites. pJY15α and its derivatives containing promoter sequences were introduced into *E. coli* DH5α by electroporation.

A qualitative α-amylase assay was done by examining the size of clear zones (halos) around colonies after staining plates containing soluble starch (Sigma, USA) with 10 mM I_2-KI solution. For a quantitative assay, a method previously described was used [13].

Results and Discussion

Screening of Promoters from *L. mesenteroides* SY2 Chromosomal DNA

pBV5030 was used to screen promoters from chromosomal DNAs of *L. mesenteroides* SY2. Insertion of a fragment upstream of a promoterless *cat*-86 was expected to confer *E. coli* host resistance against Cm if the fragment contained a promoter sequence or a sequence capable of acting as a promoter (pseudo promoter) [8]. A total of 37 *E. coli* TFs grew in the presence of 400 μg/ml Cm. Seventeen of them grew when the Cm level was increased to 1,000 μg/ml. Four TFs (03C, 04C, 25C, and 33C) even grew at the concentration of 1,250 μg/ml. When the inserts were examined by agarose gel electrophoresis, fragments with different sizes (0.3–0.7 kb) were observed. The results indicated that diverse genomic DNA fragments were recovered.

Promoter Characterization

DNA sequencing was done for 17 clones showing higher Cm resistance. The size of each fragment was determined as below: 283 bp (03C), 270 bp (04C), 313 bp (05C), 369 bp (06C), 379 bp (07C), 358 bp (08C), 415 bp (09C), 458 bp (10C), 453 bp (11C), 436 bp (13C), 532 bp (15C), 603 bp (18C), 629 bp (20C), 664 bp (21C), 406 bp (22C), 527 bp (25C), and 631 bp (33C) (Table 1). The smallest fragment was 270 bp in size and the largest one was 664 bp in size. GenBank accession numbers are from JN021454 to JN021470 for these sequences.

Analyses of the nucleotide sequences revealed that the fragments were derived from different regions on the chromosome of *L. mesenteroides* SY2. Some were non-authentic promoters (pseudo promoters) because the putative promoter sequences were located in the middle of ORFs. Others seemed to be real promoters in charge of transcription of the linked genes. For some sequences, linked genes were not found in the sequenced regions. Non-authentic promoters can also serve as promoters for expression vectors as long as they can initiate transcription of a target gene downstream of the same plasmid vector. The environments where transcription occurs on a plasmid such as pBV5030 in a heterologous host may be different from those in the genome of the original cell, *L. mesenteroides* SY2.

Determination of Transcription Start Sites

The transcription start sites of three selected promoters were determined by the primer extension method with RNA prepared from *E. coli* DH5α containing pJY04α, pJY25α, and pJY33α, respectively (Fig. 1). The transcription start site was the second A in the AAC sequence of P04C, the second A in the AAA sequence of P25C, and A in the TAT sequence of P33C (Fig. 2). The minor bands in the autoradiogram may be due to RNase activity [14]. The start site of P04C was located in the middle of an ORF for a hydroxymethylpyrimidine kinase/phosphomethylpyrimidine kinase, a bifunctional enzyme catalyzing two consecutive phosphorylation steps in the thiamine phosphate biosynthesis pathway, leading to the synthesis of vitamin B_1. The start site of P25C was also located in an ORF, 26 nucleotides upstream of the stop codon of an ORF encoding a CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase, which catalyzes the conversion of CDP-diacylglycerol and glycerol-3-phosphate to CMP and 3-(3-phosphatidyl)-glycerol 1-phosphate for the synthesis of acidic phospholipids. An ORF for RecA was located 139 nucleotides downstream from the stop codon of CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase. The start site of P33C was located upstream of an ORF for ATPase on the complementary strand. The direction of transcription by P33C was divergent from that of the ATPase gene. There was a space of 267 nucleotides between the start codon of the ATPase gene and the presumed -35 promoter sequence of P33C. No ORF was found in the 200 bp downstream region from the transcription start site.

For P03C, P05C, and P07C in Fig. 2, transcription start sites were assumed, based on the nucleotide sequences without experiments. The presumed start site of P05C was located upstream of an ORF for plasmid mobilization protein. The space between the presumed transcription start site and the first codon of plasmid mobilization protein was 136 nucleotides. The orientation of P05C indicated that this was highly likely a real promoter, responsible for the transcription of a plasmid mobilization protein gene in *L. mesenteroides* SY2. A dinucleotide (TG) was present at position -15 for 03C, 04C, 07C, and 25C, which is a known

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03C
AGCACCTGAACCATAACTAAATATGGCCAACTGCTCTCCTGCAACCAAATCTTGTGCGTAACTCAATAATGATAACAAGCTTAAA 170
04C
GTTCGCGCTAGTAAGACGGGCATGTTATCAGATGCTAGCTTAATTGAAGCAGTGGTAAAAGCTTATAAACAGTTTGACTTTGGTC 255
05C
TTGCCCCGTCACGGCGTGACCCGCCGGAGGTTTTACAAGCCGGATAAGTGGCGCTGTAAAATAGCCGTTTACCCAGTAGGAAGGG 425
GAAACGCTACGGTCATCGCTTGTCGATGACACGCCTCGCAGAGCCTGTCCAAAATATCATTTTGGTATAACAGGCTATACCAGAT 510
                                                      M  A  H  L  K  K  N  T  R  G  A
TTTTAACGGTGTTATACTGGTCTTGGAAAACCTTTTTCGAGGAGGCATTTTTTATGGCGCATTTAAAGAAAAATACGCGTGGCGC 595
 V  P  G  L  A  V  H  F  E  R  K  T  D  H  H  T  N  K  D  I  D  V  S  K  T  Y  L  N
AGTACCTGGTTTAGCGGTTCACTTTGAACGTAAAACCGATCATCATACTAACAAAGACATTGATGTGTCGAAAACCTATCTGAAT 680
07C
CCGCGTCCATTAATTTTTGTTGATAAGGTTTCTTCACCTTTCAAAACGGCCTGAGCTGTCAAGGTCGTGGCCAACAAACCACGTC 255
25C
ATTGTGGGTTGCTGTTATCTTTACAATTTATTCTGGTATCGATTATTTTTGGCAAAATAGAAATGTCTTTTCTGATGGTATGTAA 765
33C
CTTTATAAAATTATTGACATAGCGGTAGCTATTACGTTATATTATGTGCATAAACATAAATTGAGATAATTAATTTATTACAAAA 935
```

Fig. 2. Nucleotide sequences of six putative promoters from *L. mesenteroides* SY2.
Putative promoter sequences are underlined (-35 region, ·······; -10 region, ——) and transcription start sites are indicated with asterisks above the nucleotides.

feature of LAB promoters [15]. Further studies are necessary if these transcription start sites are to be used in *L. mesenteroides* SY2.

Determination of Relative Promoter Strength

In studies on promoter screening from *L. lactis*, the chloramphenicol acetyltransferase (CAT) gene was often used as a reporter to assess the promoter strength [16, 17]. However, few works have been reported for the promoter screening from *Leuconostoc* species. In this study, a reporter vector, pJY15α, was constructed using a promoterless α-amylase gene, and used to evaluate the efficacies of promoters. A 1.5 kb α-amylase gene without its own promoter was amplified from pFML1 where a 4.6 kb HindIII fragment containing the whole α-amylase gene from *Lb. amylovorus* B4540 was inserted into pBR322 [12]. The 1.5 kb fragment corresponded to the 5′ region of the α-amylase gene. It was known that the 5′ fragment still encoded an active α-amylase [12]. Putative promoter sequences were inserted upstream of the α-amylase gene in pJY15α and the resulting plasmids were named the pJY-α series (Table 1). Qualitative enzyme activities were assessed by spotting *E. coli* TFs onto a LB plate with soluble starch (Fig. 3). Big halos were observed from TFs harboring pJY-α plasmids, and *E. coli* with pJY04α showed the biggest halo (Fig. 3).

E. coli with pSJE did not produce a halo but cells with pJY15α showed a halo, indicating that transcription still occurred in pJY15α. As controls, *E. coli* TFs with different plasmid constructs were also spotted. pSJETα and pCW4Tα were derivatives of pSJE and pCW4, respectively, both

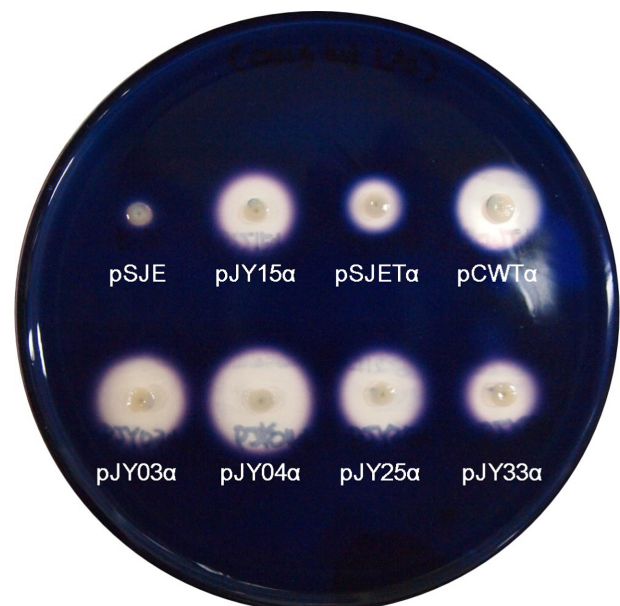

Fig. 3. Qualitative detection of α-amylase activities of *E. coli* transformants harboring various plasmid constructs.
A culture of an *E. coli* TF was spotted (1 μl) onto a LB agar plate with soluble starch (1% (w/v)). The plate was incubated overnight at 37°C and stained with 10 mM KI-I_2.

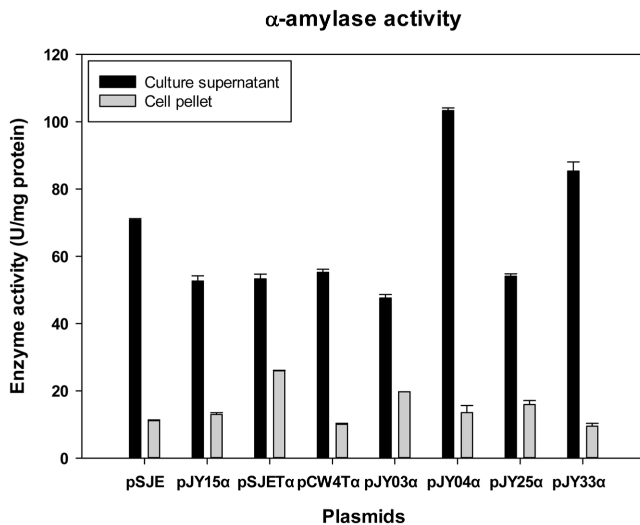

Fig. 4. α-Amylase activities of *E. coli* transformants carrying pJY-α series plasmids with putative promoters.
Enzyme activity is shown as the mean value from six measurements.

containing an α-amylase gene from *Bacillus licheniformis* [13, 18] (Table 1). α-Amylase activities of *E. coli* TFs containing pJY15α, pJY03α, pJY04α, pJY25α, and pJY33α were 66.9, 78.7, 122.1, 70.8, and 99.3 U, respectively. The activities were the sum of those of culture supernatant and cell pellet (Fig. 4). The activity of pJY04α was 1.8 times higher than pJY15α, and that of pJY33α was 1.5 times higher. The activity measurement results agreed with the qualitative detection of α-amylase activities of *E. coli* TFs in Fig. 3. The efficacies of P04C and P33C as promoters were confirmed by two different reporters, *cat*-86 and *amyL*, and both might be used for construction of an expression vector for heterologous expression of a foreign gene in LAB.

A total of 17 putative promoters were isolated from the chromosome of *L. mesenteroides* SY2 and sequenced. Three promoters conferring a higher degree of Cm resistance were tested for their efficacies in *E. coli* using a promoterless α-amylase gene from *Lb. amylovorus* as a reporter. *E. coli* cells harboring pJY04α (promoter 04C) showed the highest α-amylase activity, followed by cells harboring pJY33α (promoter 33C), when examined by qualitative plate assay and quantative activity measurements. The results indicated that these promoters worked in *E. coli*. Unfortunately, these promoters were not tested in LAB hosts and their efficacies should be verified in the future. *L. citreum* and *Lb. brevis* were transformed with pJY15a, and the transformation efficiencies were 3.2×10^1 and 2.8×10^2 transformants/μg DNA, respectively (data not shown). However, TFs harboring pJY15α derivatives containing putative promoters were not obtained, for an unknown reason. In the case of a gene whose expression causes toxic effects to host, an inducible promoter rather than a constitutive one should be used. The nisin-controlled gene expression system is a well-known example, where the *nis* promoter is induced by the addition of nisin into the culture at sublethal concentrations [20]. Expression of an α-amylase gene from *Lb. amylovorus* is not likely to cause any detrimental effect for the host. Low transformation efficiencies for LAB might be responsible, at least partially. Transformation efficiencies for *Leuconostoc* spp. and related LAB were in the range of 10^1–10^5 transformants/μg DNA, depending upon the species in our laboratory [7, 13, 19]. Clearly, the efficiencies need to be increased for more efficient gene cloning and expression studies.

Promoters like 04C and 33C might be useful for the construction of a food-grade expression vector for *Leuconostoc* ssp. or *Lactobacillus* ssp. after their efficacies in LAB are confirmed. Food-grade expression vectors must consist of food-grade selection markers and vector frames, including origins in addition to promoters. In other words, any sequence from pathogenic or potentially pathogenic organisms like *E. coli* should not be included. As a food-grade selection marker, an alanine racemase gene (*alr*) from *Lb. plantarum* WCFS1 was used in a series of expression vectors for heterologous expression of the β-galactosidase gene from *Lb. reuteri* in *Lb. plantarum* [21]. Genes conferring resistance against nisin or heavy metals such as cadmium or copper were also used as selection markers [22]. More and novel food-grade selection markers should be made available before efficient food-grade expression vectors are constructed and used for the production of valuable proteins in LAB hosts.

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