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ARTICLE

Construction of a Bile-responsive Expression System in *Lactobacillus plantarum*

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Abstract This study aimed to develop a bile-responsive expression system for lactobacilli. The promoters of four genes, encoding phosphoenolpyruvate-dependent sugar phosphotransferase (mannose-specific), L-lactate dehydrogenase (LDH), HPr kinase, and D-alanine-D-alanine ligase, respectively, which were highly expressed by bile addition in *Lactobacillus johnsonii* PF01, were chosen. Each promoter was amplified by polymerase chain reaction and fused upstream of the β-glucuronidase gene as a reporter, respectively. Then, these constructs were cloned into *E. coli-Lactobacillus* shuttle vector pULP2, which was generated by the fusion of pUC19 with the *L. plantarum* plasmid pLP27. Finally, the constructed vectors were introduced into *L. plantarum* for a promoter activity assay. The LDH promoter showed the highest activity and its activity increased 1.8-fold by bile addition. The constructed vector maintained in *L. plantarum* until 80 generations without selection pressure. A bile-responsive expression vector, pULP3-P_{LDH}, for *Lactobacillus* spp. can be an effective tool for the bile-inducible expression of bioactive proteins in intestine after intake in the form of fermented dairy foods.

Keywords bile-responsive, expression, *Lactobacillus*, plasmid

Introduction

Lactobacilli are members of lactic acid bacteria (LAB) and are generally acid- and bile-tolerant (Herbel et al., 2013; Lebeer et al., 2008; Yuki et al., 1999). They are found in a variety of fermented foods and in the gastrointestinal tract of humans and animals (Mandal et al., 2016; Valeriano et al., 2017). Some *Lactobacillus* strains have also been reported to colonize the intestinal mucosal layer and epithelium of the host (Valeriano et al., 2017, Wang et al., 2009). Therefore, intake of *Lactobacillus* strains which express bioactive proteins in intestine in the form of fermented dairy foods is very attractive (Bermudez-Humaran et al., 2013).

There continues to be great interest in the development of genetic tools for the regulation of gene expression in lactobacilli. In particular, inducible promoters have proven to be powerful tools in many biotechnological areas. Indeed, several regulated expression systems for LAB have been reported in recent years, including a heat-shock

inducible system (Maidin et al., 2014), sugar-inducible systems (Duong et al., 2011), bacteriocin-inducible systems (Axelsson et al., 2003; Sorvig et al., 2003), as well as other stresses (Benbouziane et al., 2013; Bohmer et al., 2013; Perez-Arellano and Perez-Martinez, 2003). One of the best characterised expression systems is the nisin-controlled gene expression (NICE) system in *Lactococcus lactis*, in which gene expression is activated by the addition of nisin in the culture medium (Horn et al., 2003), following which the *L. lactis* strain successfully secretes human interleukin-22 under regulation of the nisin-inducible promoter (Loera-Arias et al., 2014).

In the intestine, lactobacilli are exposed to bile, which facilitates fat absorption during digestion. Some lactobacilli can withstand the physiological concentrations of bile to survive or colonise the gut (Hamon et al., 2012; Taranto et al., 2006), and several groups have characterized gene expression in intestinal lactobacilli under gastrointestinal conditions (Alcantara and Zuniga, 2012; Chen et al., 2017; Pajarillo et al., 2015; Pfeiler et al., 2007). Previously, a gene expression system using a bile-responsive element located upstream of the bile-inducible transporter was developed in *Bifidobacterium longum* to induce protein expression in the intestinal tract (Ruiz et al., 2012). However, to the best of our knowledge, *Lactobacillus* expression system induced by bile, an intestinal signal, has not yet been developed.

Our previous proteomic study on the bile response of *Lactobacillus johnsonii* PF01 identified highly expressed proteins by bile (Lee et al., 2013). In this study, we selected promoter regions of the genes upregulated by bile and compared their bile-responsiveness using β-glucuronidase reporter gene. Herein, we report the development of a bile-responsive expression system for *Lactobacillus plantarum*.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth (Difco, Petroit, MI, USA) at 37℃ with shaking, while *Lactobacillus* strains were grown in De Man, Rogosa, and Sharpe (MRS) medium (Difco, USA) at 37℃ without shaking. *E. coli* DH5α was used as a host strain for vector

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strain		
Lactobacillus plantarum LP27	Source of plasmid pLP27	This study
L. plantarum SK156	Transformation host, erythromycin resistance negative	This study
Escherichia coli DH5α	Transformation host for cloning	Takara Bio Inc. (Japan)
Plasmid		
pUC19	pBR322 replication origin, *lacZ*, Amp^r, multicloning site	Takara Bio Inc. (Japan)
pNZ8008	Source of β-glucuronidase gene (*gusA*)	Mo Bio Laboratories (USA)
pLP27	Cryptic plasmid from *L. plantarum* LP27	This study
pULP1	pUC19 with pLP27 fragment	This study
pULP2	pULP1 with erythromycin resistance gene (Em^r)	This study
pULP3-xxx[1)]	pULP2 with promoter xxx and *gusA* gene	This study

1) xxx: promoter $EIID^{Man}$, LDH, HPrK, or DDL.

construction. When appropriate, ampicillin and erythromycin (Sigma-Aldrich, St. Louis, MO, USA) were used at concentrations of 100 μg/mL and 3 μg/mL, respectively, for selection of transformants of *E. coli* and *Lactobacillus*.

DNA isolation, transformation, and bile induction

General procedures for DNA manipulation were performed as described previously (Sambrook et al., 1989). All enzymes were purchased from Takara Bio, Inc. (Japan). *E. coli* plasmid DNA was isolated using a QIAprep spin miniprep kit (Qiagen, Valencia, CA USA) and *Lactobacillus* genomic DNA was isolated according to the method of Walker and Klaenhammer (1994).

E. coli DH5a transformation was done by heat shock method performed as described by Sambrook et al. (1989). Preparation of electrocompetent *Lactobacillus* cells and electrotransformation were done as described by Kim et al. (2005), with some modifications. *Lactobacillus* cells were grown in MRS broth supplemented with 1% glycine at 37℃ until it reached early-log phase (OD_{600}=0.2–0.3) and placed on ice for 10 min. Afterwards, they were washed twice in cold washing buffer (5 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.4), and resuspended in ice-cold electroporation buffer (1 M sucrose, 3 mM $MgCl_2$, pH 7.4). On the other hand, 1 μg of plasmid DNA was added to 50 μL of ice-cold cell suspension (~10^9 CFU/mL) in a disposable cuvette (Gene Pulser® Cuvette, 0.2 cm electrode gap; Bio-Rad, Hercules, CA, USA) and held on ice for 5 min. This mixture was subjected to electroporation using GenePulser Xcell™ (BioRad) using 200 Ω, 2.0 kV and 25 μF capacitance conditions. Finally, the cell suspension was spread on MRS agar plate supplemented with 3 μg/mL of erythromycin and then incubated at 37℃.

For bile induction experiments, recombinants were grown in MRS-erythromycin broth, harvested, and resuspended in MRS-erythromycin broth supplemented with bile (Bile bovine Sigma-Aldrich, USA).

Promoter selection

Among the genes upregulated by bile in *L. johnsonii* (Lee et al., 2013), four genes, which encode phosphoenolpyruvate-dependent sugar phosphotransferase, mannose-specific ($EIID^{Man}$), L-lactate dehydrogenase (LDH), HPr kinase (HPrK), and D-alanine-D-alanine ligase (DDL), were chosen for this study (Supplementary Table 1). The potential Pribnow box and –35 region of the promoters were predicted from the complete genome sequences of *L. johnsonii* PF01 (GenBank accession no: PRJNA67469), using BPROM and Neural Network Promoter Prediction tools at 80%–90% confidence, with a consensus length of 0.5-kb for each promoter sequence (Supplementary Table 2). Primers specific to the sequences of each promoter were designed (Supplementary Table 3) and polymerase chain reaction (PCR) amplification was performed using *Pfu* DNA polymerase with a thermal cycler (Takara, Japan) using standard procedures.

Isolation and sequence analysis of plasmid pLP27

Plasmid pLP27 (Fig. 1) was isolated from *L. plantarum* LP27 from fermented cabbage for use as the backbone plasmid. Plasmid DNA was extracted using the standard alkaline lysis method (Sambrook et al., 1989) with modifications and purified using a gel extraction kit (Qiagen, USA). Purified pLP27 was digested with the restriction enzyme SacI and cloned into pUC19 for sequencing. Nucleotide sequence similarity searches and open reading frame (ORF) analysis were performed using the BLAST program at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of bile-responsive expression vectors

The primers to clone the promoter regions for this study are listed in Supplementary Table 3. Erythromycin resistance gene

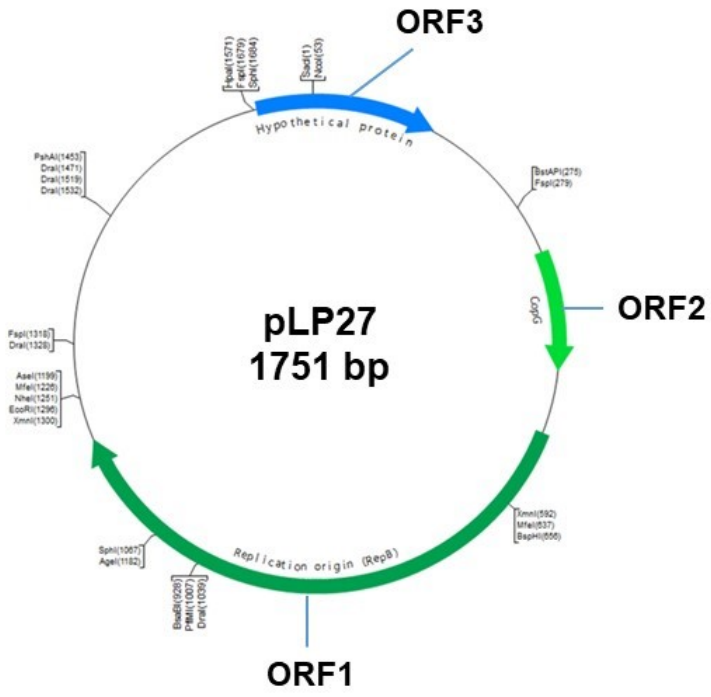

Fig. 1. Physical and genetic map of plasmid pLP27 from *Lactobacillus plantarum* LP27. Arrows indicate the length and direction of the open reading frames (ORFs). Relevant restriction enzyme sites are indicated.

as a selection marker was amplified by PCR from the genome of *L. johnsonii* PF01 with the primers Em^r-F and Em^r-R and cloned into the KpnI site of plasmid pULP1, yielding the pULP2, as shown in Fig. 2. Next, promoter sequences and the reporter gene β-glucuronidase (*gusA*) were amplified from the genome of *L. johnsonii* PF01 and pNZ8008, respectively, and fused via a second round of PCR. The resulting product was inserted into the SalI and PstI sites of pULP2, generating pULP3-xxx (xxx: P_{EIIDM}, P_{LDH}, P_{HPrK}, or P_{DDL}), which contain different kinds of promoters, respectively. Finally, the constructed plasmids were introduced into *L. plantarum* SK156 to measure promoter activity.

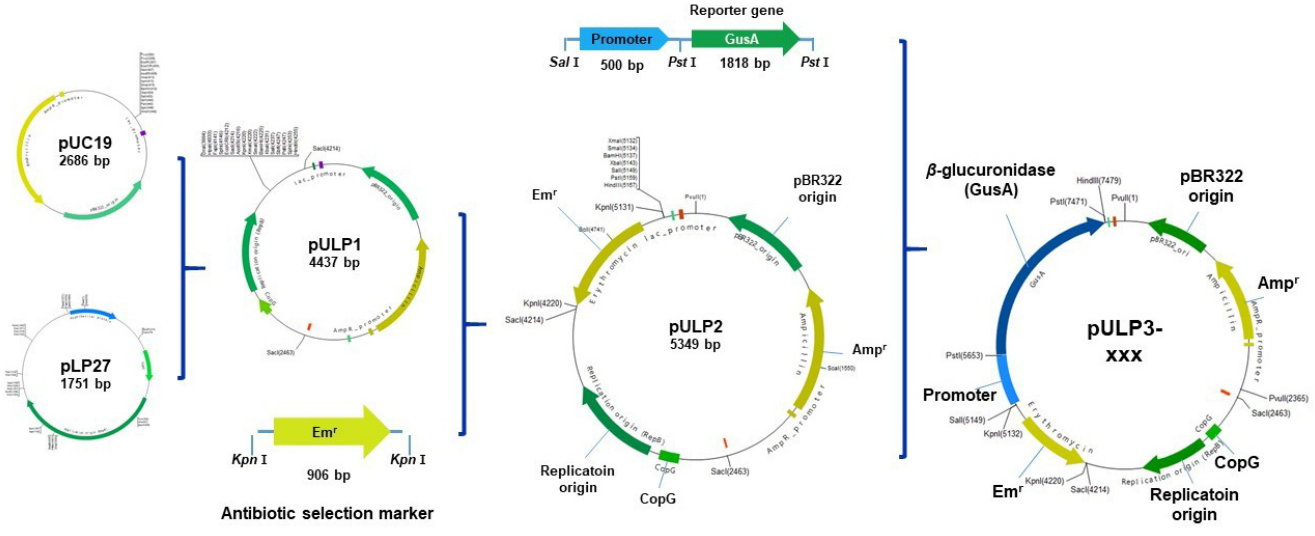

Fig. 2. Construction of bile-responsive expression vectors.

Promoter activity assay

The transformants were selected and incubated in MRS broth containing erythromycin (3 μg/mL). After growth to mid-log phase (A_{600}=0.4–0.5), cells were harvested by centrifugation at 4℃ and washed with ice-cold MRS broth. Then they were resuspended in an equal volume of MRS broth supplemented with bile (0%–0.1% (w/v)) and erythromycin (3 μg/mL), and incubated at 37℃ for 1 h. Cell-free extracts (CFEs) were harvested following cell disruption by sonication. Protein concentrations were determined using the Bio-Rad protein assay kit (Bradford, 1976). Promoter activity was assessed according to the GusA activity of CFEs, which was measured by the hydrolysis of 4-nitrophenyl β-D-glucuronide (PNPG; Sigma-Aldrich) as described previously (Platteeuw et al., 1994). For assay, CFEs were warmed to 37℃ and 200 μL of CFE was added to 800 μL of GUS buffer (100 mM sodium phosphate, 2.5 mM EDTA, and 1.0 mM PNPG, pH 6.0). After incubation at 37℃ for 5 min, 1 mL of 0.2 M Na_2CO_3 was added to stop the reaction. The units of Gus activity are described as picomoles of 4-nitrophenyl liberated per minute per milligram of protein. Three biological replicates were performed for all experiments.

Segregational stability of the plasmid vector

The segregational stability of the construct pULP3-xxx was evaluated in plasmid-free *L. plantarum* SK156. Transformed *L. plantarum* SK156 was grown in MRS medium without erythromycin for approximately 120 generations. Every 20 generations, an aliquot of the culture was collected, spread on erythromycin-free MRS agar medium, and 100 colonies were picked and replicated on MRS agar medium with and without erythromycin. Colonies grown on each medium were counted and calculated as follows: $(N_e/N)\times100$, where N_e and N are the number of colonies grown on MRS medium with and without erythromycin, respectively. Three biological replicates were performed for all experiments and data are represented as mean±SEM.

Nucleotide sequence accession number

The complete nucleotide sequence of pLP27 was deposited in the GenBank database under accession number MG944248.

Results and Discussion

Sequence analysis of plasmid pLP27

Plasmid pLP27 is a native plasmid of *L. plantarum* LP27 originally isolated from fermented cabbage. This plasmid was linearized with the restriction enzyme SacI and cloned into vector pUC19, generating pULP1. DNA sequencing of pLP27 revealed a 1,751 bp product with a GC content of 41.2% and three putative ORFs (Fig. 1). The products of the three ORFs and the most homologous protein of each identified in the GenBank database are listed in Table 2. The translated protein sequence of ORF1 showed that it shares 93% identity with the CopG family transcriptional regulator of *L. heveticus* DSM 20775. CopG is the prototype of a series of repressor proteins that are encoded by plasmids exhibiting a similar genetic structure at their leading strand initiation and control regions (del Solar et al., 1995). CopG also regulates plasmid copy number by binding the replication protein promoter for CopG (Gomis-Ruth et al., 1998; Hernandez-Arriaga et al., 2009). Therefore, ORF1 may be a transcriptional regulator for the expression of pLP27. ORF2 encoded a protein of 219 amino acids with 98% identity with the replication protein RepB of plasmid pLH2 of *L. helveticus* ATCC15009, which belongs to the rolling-circle replication plasmid family (van Kranenburg et al., 2005; Zhai et al., 2009). This indicates that pLP27 may replicate by a rolling-circle mechanism, which should be elucidated in the future. The predicted protein of ORF3 had no conserved domains or homolog sequences in the database, indicating that it is a unique, hypothetical protein. Plasmid pLP27 combined with pUC19, namely pULP1, was

used as a backbone for the construction of bile-responsive expression vectors.

Selection of promoters and construction of bile-responsive expression vectors

Our previous proteomic analysis of *L. johnsonii* PF01 revealed some proteins were upregulated by bile (Lee et al., 2013). Among the genes upregulated above two-fold at protein level by bile, genes encoding $EIID^{Man}$, LDH, HPrK, and DDL were selected and their promoters were used to construct bile-responsive expression vectors (Supplementary Table 1). Fig. 2 shows the cloning steps for construction of the pLP27-derived bile-responsive expression vectors. First, the erythromycin resistance gene from *L. johnsonii* PF01 was cloned into the KpnI site of pULP1, generating pULP2. The promoter regions of four genes were amplified, respectively, from the genome of *L. johnsonii* PF01 and fused upstream of the *gusA* reporter gene. Then, these constructs were cloned into the SalI and PstI sites of pULP2, generating pULP3-P_{EIIDM}, pULP3-P_{LDH}, pULP3-P_{HPrK}, and pULP3-P_{DDL}, respectively (Fig. 2).

Comparison of promoter activity and bile-responsiveness

Once all of the constructs were obtained, they were introduced by electroporation into *L. plantarum* SK156 (Table 1), and GusA activity was measured in different bile concentrations (Kim et al., 2006). The promoterless vector, which contains the *gusA* gene only was used as a negative control. Transformants were grown to early stationary phase either in the presence or absence of bile. Promoter activity was analysed by measuring GusA activity using PNPG as a substrate. As shown in Fig. 3,

Table 2. ORF analysis of pLP27 from *Lactobacillus plantarum* LP27

ORF	Function	Position	% identity	Best BLAST match	GenBank accession no.
ORF1	CopG family transcriptional regulator	329–469	93	*Lactobacillus helveticus* DSM 20775	WP_003627912.1
ORF2	Replication protein RepB	542–1,201	98	Plasmid replication protein of pLH2, *Lactobacillus. helveticus* ATCC 15009	WP_003627911.1
ORF3	Hypothetical protein	1,681–76	98	*Lactobacillus plantarum*	WP_063723612.1

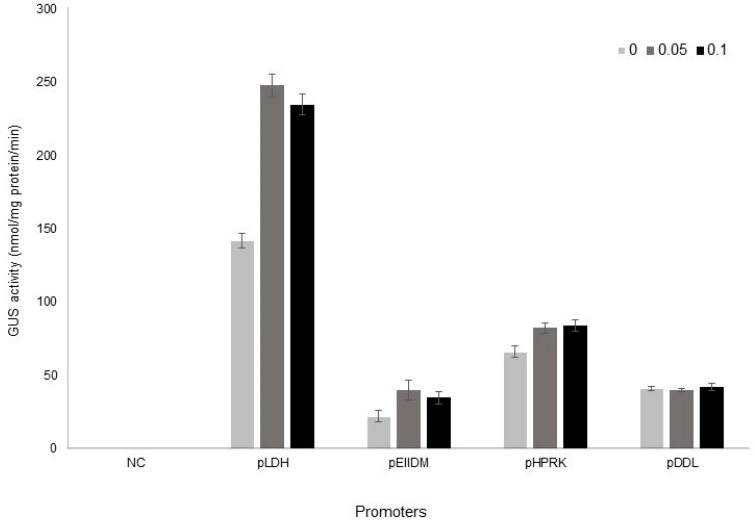

Fig. 3. β-Glucuronidase activity of protein extracts of *L. plantarum* SK156 harbouring pULP3-xxx (xxx: P_{LDH}, P_{EIIDM}, P_{HPrK}, or P_{DDL}). The bars indicate activities in the absence (light grey) and in the presence of 0.05% (dark grey) and 0.1% (black) bile. Error bars represent the standard error of the means for three independent experiments.

the LDH promoter showed the highest β-glucuronidase activity in *L. plantarum* and its activity increased 1.8-fold by bile addition. EIIDM and HPRK promoters were also inducible by bile, although their activities were lower than LDH promoter. On the other hand, DDL promoter was not responsive to bile.

Interestingly, the expression of EIIDM was more inducible by bile than that of LDH in *L. johnsonii* (Supplementary Table 1). A report by Ruiz et al. (2012) showed that a bile-responsive expression system responded differently according to bifidobacterial species, suggesting that host difference might cause different expression level. In addition, we cannot exclude the possibility that the bile responsiveness of these promoters also varies according to bile composition, because arabinofuranosidase activity as a reporter in *Bifidobacteria* was strongly induced by cholate and conjugated cholate, but not by other bile salts (Ruiz et al., 2012). The activity of promoters used in this study according bile salts also need to be elucidated in the future.

Interestingly, GusA activities in all constructs was observed without bile, although their levels except for DDL promoter were lower than in the presence of bile. It indicates that the promoter's strength can be regulated by other factors, too.

Segregational stability of pULP3-P_{LDH} in *L. plantarum*

pULP3-P_{LDH} showing the highest GusA activity was used to investigate segregational stability in the *L. plantarum*. The transformation efficiency was 1.2×10^4 CFU/μg DNA in *L. plantarum* SK156 (data not shown). It was also successfully introduced into *L. fermentum*. However, no transformants of pULP3-P_{LDH} were obtained from *L. acidophilus*, *L. johnsonii*, and *L. reuteri* (data not shown). These results indicate that this vector has a limited host range. After selection of recombinant transformants, the stability of pULP3-P_{LDH} in *L. plantarum* SK156 was monitored over 120 generations of growth in MRS broth. No loss of the plasmid was observed until 80 generations in the absence of erythromycin, and plasmid possession ratio decreased gradually to 63% after 120 generations (Fig. 4), indicating relatively high stability of the plasmid vector without selection pressure.

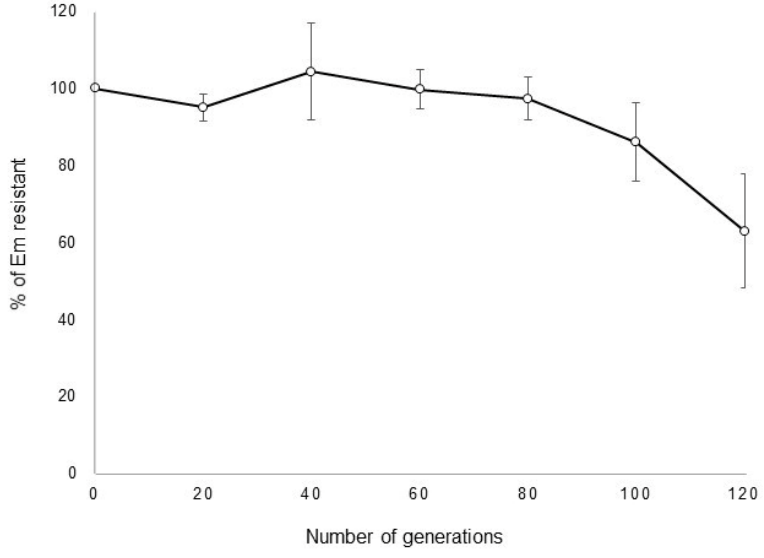

Fig. 4. Segregational stability of the vector pULP3$_{LDH}$ in *L. plantarum* SK156. *L. plantarum* SK156 harbouring the plasmid was cultured without selection pressure, plated on the same medium, and verified for plasmid maintenance by replica-plating onto erythromycin-containing MRS medium at approximately 20 generation intervals until 120 generations. *Em*, erythromycin. Error bars represent the standard error of the means for three independent experiments.

Conclusion

Although there continues to be great interest in the development of genetic tools for the regulation of gene expression in lactobacilli, to the best of our knowledge, a bile-responsive expression system in lactobacilli has not yet been developed. Herein, we report the development of a bile-responsive expression system for *Lactobacillus plantarum*. This system can be an effective tool for the expression of bioactive proteins in intestine after intake of the *Lactobacillus* spp. in the form of fermented dairy food.

Conflicts of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgements

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Author's Contributions

Conceptualization: Kang DK. Data curation: Chae JP, Pajarillo EA. Formal analysis: Chae JP, Hwang IC. Methodology: Chae JP, Hwang IC. Software: Chae JP, Pajarillo EA. Validation: Chae JP. Investigation: Kang DK. Writing - original draft: Chae JP, Kang DK. Writing - review & editing: Chae JP, Pajarillo EA, Hwang IC, Kang DK.

Ethics Approval (IRB/IACUC)

This article does not require IRB/IACUC approval because there are no human and animal participants.

Supplemenrary Materials

Supplementary materials are only available online from: https://doi.org/10.5851/kosfa.2018.e58.

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Supplementary Material

Supplementary Table 1. Proteins selected to use their promoters for the construction of bile-responsive expression vectors

Protein name	Promoter name	Fold change of expression level (0.1% bile/0% bile)
Phosphoenolpyruvate-dependent sugar, phosphotransferase, mannose-specific	P_{EIIDM}	13.5
HPr kinase	P_{HPRK}	5.8
L-Lactate dehydrogenase	P_{LDH}	2.1
D-Alanine-D-alanine ligase	P_{DDL}	2.1

Supplementary Table 2. Promoter region's sequences of four candidate genes

Gene's name	Promoter region's sequences (5' to 3')
Phosphoenolpyruvate-dependent sugar phosphotransferase, manose-specific, (*EIIDM*)	ATGTTAGTTCGTACTATTTCTGTTGCCATTGTTCATATTATGGATAGAAAAGCAGAAGAAGCT AACTGGAGAGCTATTGACCGTTGGCAAATGATTGCTGTTGCTTTACAAGGTTTACGAATTGC TATTCCAGCTGCATTATTACTTGCTATTCCAGCTTCTGTTGTTCGTGCTGGCTTAAATGCAATG CCAGCATGGTTAAACGAAGGTATGACCATCGGTGGTGGTATGGTTGTTGCCGTTGGTTACGC TATGGTTATTAATATGATGGCTAGCCGTGAAGTATGGCCATTCTTCATCATTGGTTTCTGTTTG GCAGCCGTTAAAGAATTAACTTTGATCGCTCTTGGTGGTATTGGTATTTCCCTTGCTATTATGT ACCTTGCTCTTGAAGGTTCTAAGGGTGGAAACAACAATAACTCTAATTCTGGTTCAGGCGAC CCATTGGGCGACATCCTGGATGATTATTAAAATTTGAGCGAGAGGAGGCTAATTATATAATG –35 –10 RBS
HPr kinase (*hprK*)	TTGTTACCTCATCGCAGGCTCAGCGATCCCAATTGCAAAATAGAGAAACTGCAATGAATATG TTACGGGCAAAGTTATTCCAGCTAGAAGAAGAGAAGAAAAGAAAGCAGACAGAAGAACTT AAAGGTAATCAAAAAGAAATTGGCTTTGGTTCTCAAATTCGCTCTTATGTTTTTCATCCTTAC AATATGGTAAAAGATCATCGGACTAATTTTGAAAGCAGTGATGTAAATGGTGTAATGGATGG AAAATTAGATAATTTTATTTATGCTTATTTACAGTGGCTATTGAGCCAAAAAAATCCGAATTAA AGTTTAAGGAAAAGATCTATGGGATTTGTAACTATTTTAATATTGGTATTAGCTGCTTTAGTGA TTTTAAGTATTTTATTTTCAATATTTAAATTCTTTATTGGCTTGTTACCTGTTGCTATAATTGTT –35 –10 GCTTTGATTATTTGGCTACTGGCTAAGTTTTCTGGTAACAAAAAAGATACAGAAAAAACTGA TAGCCATAATCCAGTGCAGCCATCCACTAATAATCAAAAGCGAAAGAAAGCACGAAATGTTT CAGTCAAAGATGTAGAAGATAAAGAGGATAACAAAAATG RBS
Lactate dehydrogenase (*ldh*)	CATTTGCACGAATTCTAATCTTTCCAATTGGCATGTCCATATCATCATGAATAACTAAAATATC TTCAGGCTTAATTTTAAAGAAATTAGCTACTTGCGCGATTGATTTTCCCGAATCGTTCATAAA AGTCTGTGGTTCAAGAAAAATTACATCTTCACCGTTGATCTTTTGTTTTGTCCATAAGCCTTC AAACTTCTCCTTATCTAGGGTTAAACCATTTTTCTCTAAGTAATGATCTAATGCCATAAAACCT GTATTATGCTTAGTCTGATCATATTTTCTACCTGGATTTCCTAAACCTGCAATTAACTTCATTCT TTTTCATCCTCTTTGATAAATATTTCATTCTAAATCTGCTTATTGCTCTTGAGATTATAGCACAC GCTTACTTACAAATAATTAATTAGATCTTTAAAATTATATTTTTAATTTTTAAATT –35 CCAATATGATATAATCGTTATTGTTAAATAAATCACTAATATGGGAAGGAAGTATATG –10 RBS
D-Alanine-D-alanine ligase (*ddl*)	GTGATTTGAGCGTTATCATTATCGGTTTTATTTGTTGAAATACTTGGTAAGTCAAATAAAGCTC GGTAAAAATTAATATAATCTAATTGAGAATTGATATAAGAATTTGTAACAGAACCGGGTGAAA AGGGAGCCGTGAGATGAGGAGTGCTGGCATAAAGATTATCTGCATTAAAATTTTCTTTAGGT AAATTTTTATATTGATTTTGAATTTGATGAACTTCTTGAATTTCATCTTTAGTAAAGGTTGCAG CCTGTGCGTTAGTAACTATAACGGTAGAAGTAGTTGTACTTAACACAATAGTTGCTAAAAAA GTGGCTGCTTTTTTGGTAAAAGTCATAATTACTCCTTTTAGCGAATCATATAGTAGCATGATAG TAATATATATAACGTAATTATAAAAAATTGCATTCTTTTTAGACTTTTTTTCGAAATTGA –35 –10 TGCATTATAGCGTCACGATTGTTTATAATAAGGTTTCGAGGACAAAAAGGAGATAAAAATATG RBS

RBS, ribosome binding site

Supplementary Table 3. Primers to clone the genes for this study

Gene name	Primer name and sequence (5' to 3')	Restriction endonuclease site	Sources
Phosphoenolpyruvate-dependent sugar phosphotransferase, manose-specific, (*EIIDM*)	P_{EIIDM} –F: AAC GTC GAC ATG TTA GTT CGT ACT ATT TC P_{EIIDM} –R: AAC CTG CAG TAT ATA ATT AGC CTC CTC TC	*Sal*I *Pst*I	*L. johnsonii* PF01
HPr kinase (*hprK*)	P_{HPRK} –F: AAC GTC GAC GAA AGC AGA CAG AAG AAC TT P_{HPRK} -R: AAC CTG CAG TAT CCT CTT TAT CTT CTA CA	*Sal*I *Pst*I	*L. johnsonii* PF01
Lactate dehydrogenase (*ldh*)	P_{LDH} –F: AAC GTC GAC CAT TTG CAC GAA TTC TAA TC P_{LDH} -R: AAC CTG CAG ATA CTT CCT TCC ATA TTA GT	*Sal*I *Pst*I	*L. johnsonii* PF01
D-Alanine-D-alanine ligase (*ddl*)	P_{DDL} –F: AAC GTC GAC GAG CGT TAT CAT TAT CGG TT P_{DDL} –R: AAC CTG CAG ATT TTT ATC TCC TTT TTG TC	*Sal*I *Pst*I	*L. johnsonii* PF01
Erythromycin resistance gene (Em^r)	Em^r –F: GCT CGG ATC CTT TCG CAG TAA CTC TAT TAT Em^r –R: TGT AGG ATC CGC ACC CCT TTA ACT TTA TCT	*BamH*I *BamH*I	*L. johnsonii* PF01
β-Glucuronidase gene (*gusA*)	gusA-F: AAC CTG CAG ATG TTA CGT CCT GTA GAA AC gusA-R: AAC CTG CAG TCA TTG TTT GCC TCC CTG CT	*Pst*I *Pst*I	pNZ8008

Restriction sites are underlined in primer's sequences.