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Construction of a Lactococcal Shuttle/Expression Vector Containing a β-Galactosidase Gene as a Screening Marker

Han, Tae Un[1], Do-Won Jeong[1], San Ho Cho[1], Jong-Hoon Lee[2], Dae Kyun Chung[3], and Hyong Joo Lee[1,*]

[1]School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea
[2]Department of Food Science and Biotechnology, Kyonggi University, Suwon 442-760, Korea
[3]Department of Genetic Engineering, KyungHee University, Suwon 449-701, Korea

A new lactococcal shuttle/expression vector for lactococci, pWgal13T, was constructed using a β-galactosidase gene (*lacZ*) from *Lactococcus lactis* ssp. *lactis* ATCC 7962 as a screening marker. The pWgal13T was introduced into *Escherichia coli* DH5a and *L. lactis* MG1363, and was easily detected by the formation of blue colonies on a medium containing X-gal without any false transformants. Also, the quantitatively *lacZ* activity of pWgal13T was measured in *L. lactis* ssp. *cremoris* MG1363, and was found to be four times higher than that of *L. lactis* ssp. *lactis* ATCC7962 grown on a medium containing glucose, which shows that the *lacZ* gene of pWgal13T can be used for the efficient screening of *L. lactis* on general media. The pWgal13T was equipped with a lactococcal replicon of pWV01 from *L. lactis* Wg2, the new promoter P13C from *L. lactis* ssp. *cremoris* LM0230, multiple cloning sites, and a terminator for the expression of a relevant gene. The vector pWgal13T was used for the expression of the EGFP gene in *E. coli* and *L. lactis*. These results show that the lactococcal expression/shuttle vector constructed in the present study can be used for the production of foreign proteins in *E. coli* and *L. lactis*.

Key words: Lactococcal shuttle/expression vector, *Lactococcus lactis*, β-galactosidase, *lacZ*, P13C, EGFP protein

INTRODUCTION

Lactococci are a group of lactic acid bacteria known as GRAS (generally regarded as safe) organisms that are used in food production. The development of a wide variety of cloning systems has made it possible to genetically modify *Lactococcus lactis* strains for use in various industrial applications such as dairy products, fermented foods, probiotics, and live vaccines [3]. These genetically improved lactococci and their products are potentially highly useful in the production of foods or even medicines. Until now, latent applications of lactococci have been developed [4, 14, 15], with an important research topic being the construction of useful vectors such as a shuttle vector for recombinant DNA techniques [1, 14]. However, a disadvantage of most of these vectors is that several experiments must be performed to obtain reliable transformants. Screening methods depending only on antibiotic resistance as a screening marker increase the severity of this problem because such methods would probably cause spontaneous mutations that would make confer a tolerance to antibiotics on cells [18]. There have been diverse attempts to solve this problem, but simple and convenient screening systems exist for only a few lactococci vectors.

We have previously characterized the *gal/lac* operon including the β-galactosidase gene (*lacZ* gene) from *Lactococcus lactis* ssp. *lactis* ATCC 7962 [10-12, 16]. The plasmid containing the *lacZ* gene led to the formation of blue colonies on plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) that are easily observed with the naked eye. We have also characterized the promoter from *L. lactis* ssp. *cremoris* LM0230 [9] for the expression of a relevant protein in *L. lactis*. Based on these results, this study aimed to develop a useful screening marker for lactococci and to construct a lactococcal shuttle vector using the *lacZ* gene from *L. lactis* ATCC 7962 [11] as a screening marker.

*Corresponding author
Tel. 82-2-880-4853, Fax: 82-2-873-5095
E-mail: leehyjo@snu.ac.kr

MATERIALS AND METHODS

Bacterial Strains and Growth Culture

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* DH5α, *L. lactis* ATCC 7962, and *L. lactis* MG1363 were used as a host for vector construction, as a donor strain of *lacZ* gene (NCBI accession number: U60828), and as a host for EGFP gene expression under promoter P13C (accession number: AY831654), respectively.

E. coli and *L. lactis* were grown in LB broth and in M17 broth (Difco, Sparks, MD, USA) supplemented with appropriate sugar, respectively. *L. lactis* harboring recombinant plasmid containing *lacZ* as a screening marker was detected on M17 containing X-gal (4 μg/ml). The following antibiotics were employed: ampicillin (100 μg/ml) and erythromycin (200 μg/ml) for *E. coli*, and erythromycin (5 μg/ml) for *L. lactis*. *E. coli* was transformed according to the method of Hanahan and Meselson [6], and *L. lactis* was transformed by electroporation as described by Holo and Nes [8].

PCR and DNA Analysis

PCR primers were designed on the basis of known DNA sequences, and relevant restriction enzyme sites were introduced when needed (Table 2). All the PCRs comprised 35 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 2 min, and elongation at 72°C for 3 min with a commercial PCR system (GeneAmp 2700, Foster, CA, USA), using standard procedures in reaction conditions recommended by the manufacturer of Ex taq polymerase (Takara, Kyoto, Japan).

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

Strain and plasmid	Relevant characteristics	Origin or reference
E. coli DH5α	Plasmid-free, Lac^-	
L. lactis MG1363	Plasmid-free derivative of NCDO712, Lac^-, Mel^-	[5]
L. lactis ATCC 7962	Donor strain for the cloning of *lacZ*	[11]
pUC18	Am^r, *E. coli* cloning vector	NEB
pEGFP	Am^r, *E. coli* cloning vector containing EGFP gene	Clontech
pMG36e	Em^r, lactococcal expression vector	[17]
pβgal	Am^r, pUC18 derivatives carrying *lacZ*	This work
p13C	Em^r, Cm^r, donor plasmid of promoter 13C	[9]
p13βgal	Am^r, pβgal derivatives carrying promoter 13C	This work
pWgal1	Am^r, Em^r, lactococcal/*E. coli* shuttle vector, p13βgal derivatives carrying broad-host replicon of pWV01	This work
pWgal13T	Am^r, Em^r, pWgal1 derivatives carrying expression element-promoter, MCS, and terminator	This work
pWgal13TE	Am^r, Em^r, pWgal13T derivatives carrying EGFP gene	This work

Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Am^r, ampicillin resistance; *lacZ*, β-galactosidase gene; NEB, New England Biolabs; Clontech, BD Biosciences Clontech

Table 2. Primers used in this study.

Primer	Oligonucleotide Sequence (5' → 3')	Specificity	Reference
lacZ-F	GATGAGTCGACCTGAAAGGAATGGCTATGA	*lacZ*	[11]
lacZ-R	ACCGTCTGCAGAACCTGGATTTTCTCTATA	*lacZ*	[11]
P13-3	AAAACTGCAGCTCGGTACCCGGG	P13C	[9]
P13-4	AAAAGACGTCGCAGTAATTTATAAA	P13C	[9]
PMT1	CCCCGTCGACCTCGAGCATCGATCGGA	MCS	This study
PMT2	TAAATTATCGCGACCCGGATCCGATCG	MCS	This study
PMT3	AAAGGTGATTTTTTTTATAAATTATC	TpepN	NCBI accession no. M87840
PMT4	AATAAAAAAACCACCTCTAAAAGGTGA	TpepN	NCBI accession no. M87840
PMT5	GGGGCTGCAGTAATTTATAAATAAAAAAACC	TpepN	NCBI accession no. M87840
EGFP-F	CCGCTCGAGACAGGAAACAGCTATGAC	EGFP gene	This study
EGFP-R	CCGCTCGAGCTTTACTTGTACAGCTCG	EGFP gene	This study

Underlining indicates nucleotides participating in restriction sites.

Construction of pWgal Vector Series

The constructed plasmids were verified by enzyme digestion and DNA sequencing. The fragment containing the *lacZ* was amplified using primers lacZ-F and lacZ-R designed on the basis of the *lacZ* sequence from *L. lactis* ATCC 7962 (accession number: U60828). The chromosomal DNA of *L. lactis* ATCC 7962 was used as a template. The amplicon was digested with SalI and PstI, and then inserted into the same site of pUC18, and was designated pβgal1. To express the *lacZ* gene, pB13C [9] was digested with SmaI and SalI, and promoter P13C was obtained. The digested fragment was inserted at the same sites of pβgal1, and the resulting plasmid was designated p13βgal.

p13βgal was digested with SmaI and PstI, the fragment containing the *lacZ* gene and P13C, and then the resulting fragment was inserted into pMG36e [17], thus resulting in pWgal1.

Primers were synthesized to construct the double strand containing multiple cloning sites (MCS) and the terminator of lactococcal aminopeptidase N (TpepN) (accession number: M87840) (Table 2). Their construction is presented schematically in Fig. 1. The oligomers PMT1 containing SalI and PMT2 contain the complementary sequence at the 3 end, connected by PCR. The fragment was extended using PMT1 and PMT3, PMT1 and PMT4, and PMT1 and PMT5 containing PstI from the PCR1, PCR2, and PCR3 products as a template, respectively. The resulting amplicon was equipped with MCS (*Sal*I, *Xho*I, *Cla*I, *BamH*I, and *Nru*I) and TpepN. The amplicon was digested with SalI and PstI, and was inserted at the same sites of pB13C [9], thus resulting in p13MT.

The gene fragment containing P13C, MCS, and TpepN in tandem was amplified from p13MT with primers P13-3 and P13-4 containing PstI and AatII, respectively. The amplicon was digested with PstI and AatII, and inserted into the same site of pWgal1. The resulting expression/secretion vector was named pWgal13T (Fig. 2).

To identify the expression ability of pWgal13T, an enhanced green fluorescent a protein gene was amplified with primers EGFP-F and EGFP-R containing XhoI restriction enzyme sites from pEGFP (BD Biosciences Clontech, Mountain View, CA, USA). The amplicon was digested with XhoI, and inserted into the same site of pWgal13T, resulting in plasmid pWgal13TE.

β-Galactosidase Activity Assay

The β-galactosidase activity was determined as described by Chang *et al.* [2]. Briefly, an overnight culture of *L. lactis* transformant cells in M17 medium was inoculated into M17 medium containing 0.5% glucose or 0.5% lactose,

Fig. 1. Method of production of fragments containing MCS (italic) and TpepN (boldface).

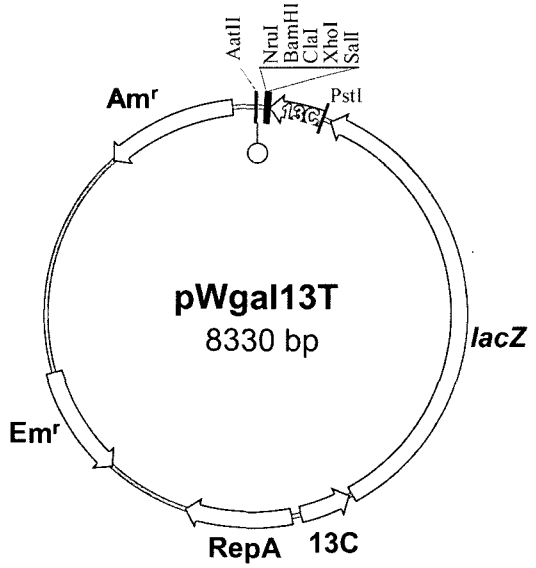

Fig. 2. Map of the expression/shuttle vector pWgal13T. Symbols: , terminator of *pepN*; Am^r, ampicillin resistance; Em^r, erythromycin resistance.

and incubated for 15 h at 30°C. One milliliter of the cells was harvested by microcentrifugation at 5000 rpm for 5 min, washed twice with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 5 mM β-mercaptoethanol; pH 7.0), and resuspended in Z-buffer. The resuspended cell volume was adjusted to 1 ml and then mixed vigorously with 50 ml of SDS (0.1%) and 20 ml of chloroform. This mixed solution was allowed to stand for 5 min at 28°C, mixed well with 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml), and placed in a 30°C water bath. When a yellow color developed, 1 ml of 1 M Na_2CO_3 was added to stop the enzyme–substrate reaction. The absorbance of the supernatants produced by microcentrifugation of this solution was measured at 420 and 550 nm, and the activity was calculated as $1{,}000(A_{420}-1.75A_{550})/(tvA_{600})$, where t is the reaction time in minutes and v is the volume in milliliters of the culture used in the assay.

Confocal Laser Scanning Microscopy

For microscopy, cells were individually placed onto glass slides and carefully fixed with cover slips. The fluorescence was measured using a UV confocal scanning laser microscope (MRC1024, Bio-Rad, Hercules, CA, USA). For EGFP detection, an argon laser excited the specimen at 488 nm, and fluorescence was monitored through a 30-nm-wide emission filter centered at 515 nm.

RESULTS AND DISCUSSION

To develop the lactococcal expression/shuttle vector pWgal13T using a *lacZ* gene as a screening marker, a series of plasmids based on the vector pMG36e [17] containing a broad-host-range replicon of pWV01 [13] was constructed (Fig. 2), which enabled the easy genetic manipulation in *E. coli* and *L. lactis*. Also, the pWgal13T was composed of the *lacZ* gene from *L. lactis* ATCC 7962 as a screening marker, P13C as a promoter from *L. lactis* LM0230, TpepN from *L. lactis* MG1363 as a terminator, and MCS. P13C, TpepN, and MCS were inserted for the easy cloning and expression of relevant proteins.

The promoter P13C from *L. lactis* LM0230 [9] – except the P13C for a foreign protein – was located upstream of the *lacZ* gene on pWgal13T. To quantitatively determine the *lacZ* activity as a screening marker under promoter P13C, the β-galactosidase activity of *L. lactis* MG1363 (pWgal13T) was measured after growing at 30°C in the presence of lactose and glucose (Table 3). The β-galactosidase activity of *L. lactis* ATCC 7962 (from which the *lacZ* gene originated) revealed that lactose-grown cells showed 23-fold higher activity than cells grown on glucose. This result suggests that *lacZ* was induced by lactose via the putative promoter controlled by lactose in the *gal/lac* operon being subject to a *trans*-element carbon catabolite control protein [7]. However, the β-galactosidase activity of lactose-grown *L. lactis* MG1363 (pWgal13T) was similar to that of glucose-grown cells. This result suggests that *lacZ* was constitutively expressed under P13C, which was characterized as a constitutive promoter [9]. Also, the absence of detectable activity in the parental strains *L. lactis* MG1363 and *L. lactis* MG1363 (pMG36e) clearly indicates that *lacZ* is responsible for the activity of β-galactosidase shown in the transformant. In addition, the *lacZ* gene as a screening marker of the pWgal13T led to the formation of blue colonies on plates supplemented with X-

Table 3. β-Galactosidase activity of *L. lactis* MG1363 harboring pWgal13T.

	Glucose	Lactose
L. lactis MG1363	ND	ND
L. lactis MG1363 (pMG36e)	ND	ND
L. lactis MG1363 (pWgal13T)	48 ± 3	53 ± 4
L. lactis ATCC7962	13 ± 1	300 ± 5

L. lactis MG1363 harboring pWgal cells was grown on M17 broth containing glucose (0.5%) or lactose (0.5%). ND, not determined.

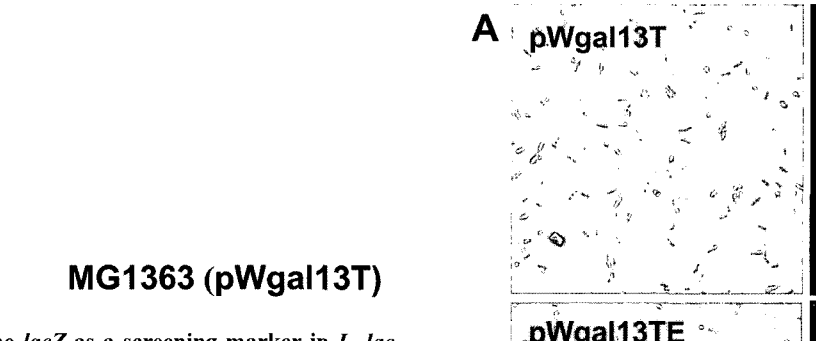

Fig. 3. Evaluation of the *lacZ* as a screening marker in *L. lactis*. The β-galactosidase activity of the *L. lactis* transformants containing pWgal13T was determined on a M17 plate containing X-gal.

gal (Fig. 3). Taking advantage of this feature offered by the *lacZ* gene, direct screening during the construction of pWgal13T was achieved in *L. lactis* MG1363 by the formation of blue colonies on plates containing X-gal. Consequently, this phenotype was easily recognizable on plates containing X-gal.

To evaluate the use of the new vectors in gene cloning and expression, pWgal13TE containing the EGFP gene as a reporter gene under the P13C of pWgal13T and upstream of the TpepN was used in *E. coli* DH5α and *L. lactis* MG1363. The expressions of the EGFP gene of cells containing pWgal13TE were determined using a confocal laser scanning microscope (Fig. 4). *E. coli* (pWga13T) was not a fluorescent strain, whereas *E. coli* (pWgal13TE) was fluorescent due to the EGFP activity. The same results were obtained in *L. lactis*. This suggests that pWgal13T successfully produced the expression of EGFP in *E. coli* and in *L. lactis*.

Here we have demonstrated the construction and evaluation of a convenient lactococcal expression/shuttle vector based on b-galactoside metabolism. The nucleotide sequence of the developed vector is known, and consists of a promoter, MCS, and a terminator, indicating that it could be used for the improvement of lactococci by self-cloning. This would allow the further development of lactococci as acceptable hosts for the production of various proteins, peptides, or metabolites. Also, the *lacZ* gene could be used as a screening marker of a food-grade vector for *L. lactis*, since transformations could be easily distinguished by the formation of blue colonies associated with â-galactosidase on media containing X-gal.

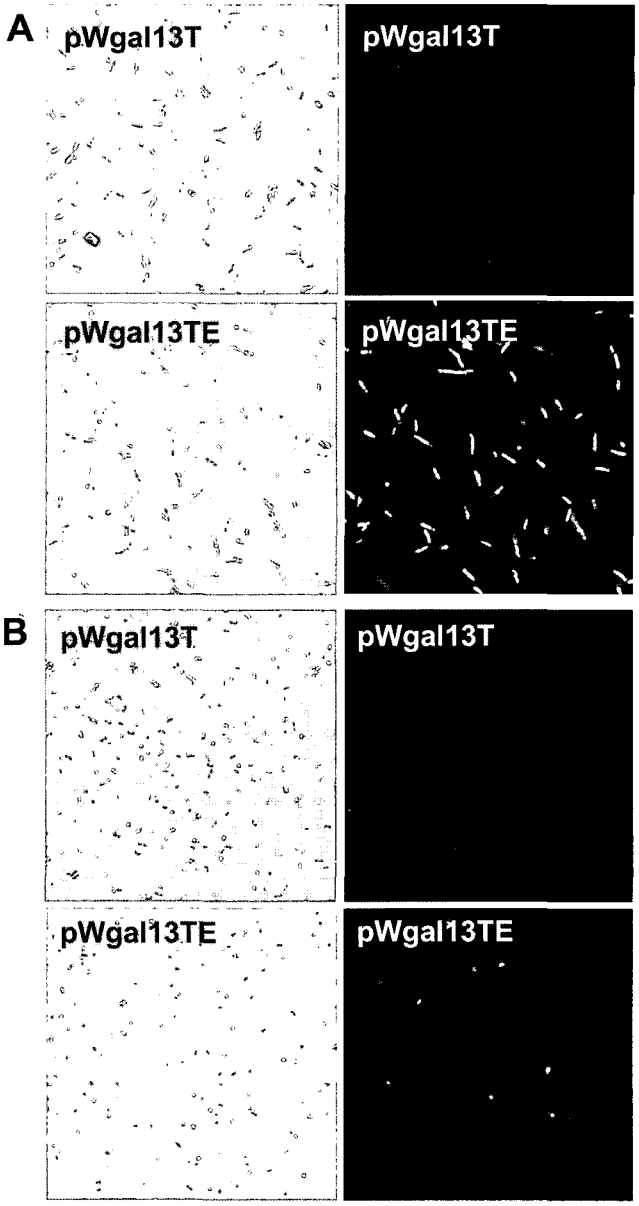

Fig. 4. The influence of promoter 13C in pWgal13T on the EGFP gene expression in *E. coli* and *L. lactis*. The β-galactosidase activity of the cells containing pWgal13TE was determined using a confocal laser scanning microscope in *E. coli* DH5a (A) and *L. lactis* MG1363 (B).

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선별마커로써 β-Galactosidase 유전자를 포함한 *Lactococcus*용 셔틀/발현 벡터 제조

한태운 · 정도원 · 조산호 · 이종훈[1] · 정대균[2] · 이형주*
서울대학교 농생명공학부, [1]경기대학교 식품생물공학과, [2]경희대학교 유전공학과

선별마커로써 *Lactococcus lactis* ssp. *lactis* ATCC 7962 유래의 β-galactosidase 유전자를 포함하는 *Lactococcus*용 셔틀/발현 벡터 pWgal13T를 제조하여 *Escherichia coli* DH5α와 *L. lactis* MG1363내로 도입하였다. 이들 형질전환체들은 X-gal을 포함하는 배지에서 파란색의 표현형을 보임으로써 쉽게 확인할 수 있었다. 또한, *L. lactis* MG1363 형질전환체로부터 β-galactosidase 활성을 측정한 결과 기존에 β-galactosidase를 활성을 지닌 *L. lactis* ATCC 7962에 비해 glucose를 포함하는 M17 배지에서 4배 정도 높은 활성을 보임으로써 선별마커로써의 효율성을 나타내었다. pWgal13T는 β-galactosidase 유전자 외에 *L. lactis* Wg2유래의 replicon과 외래 유전자의 발현을 위한 *L. lactis* ssp. *cremoris* LM0230의 promoter P13C, terminator를 포함하고 있다. 이 벡터의 이용가능성을 확인하기 위하여 외래 유전자 EGFP 유전자를 P13C 아래에 삽입하여 *E. coli*와 *L. lactis*에서 발현을 확인하였다. 이 연구에서 제조된 *Lactococcus*용 발현 벡터 pWgal13T는 *E. coli*와 *L. lactis*에서 외래 유용 유전자를 생산을 위해 이용 할 수 있을 것이다.

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