J Food Sci Nutr
Vol 13, p 190~195 (2008)
DOI: 10.3746/jfn.2008.13.3.190

J Food Science and Nutrition

Expression of Alpha-Amylase Gene from *Bacillus licheniformis* in *Lactobacillus brevis* 2.14

Kang Wook Lee[1], Ji Yeong Park[1], Gyoung Min Kim[1], Gun-Hee Kwon[1], Jae-Yong Park[1], Mee-Ryung Lee[1], Jiyeon Chun[3], and Jeong Hwan Kim[2†]

[1]*Division of Applied Life Science (BK21 program), Graduate School and*
[2]*Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 660-701, Korea*
[3]*Department of Food Science and Technology, Sunchon National University, Sunchon 540-742, Korea*

Abstract

The α-amylase gene, *amyL*, from *Bacillus licheniformis* was expressed in *Lactobacillus brevis* 2.14 and *Escherichia coli* DH5α using two different shuttle vectors, pCW4 and pSJE. *E. coli* transformants (TFs) harboring either pCW4Tα or pSJETα produced active α-amylase but *L. brevis* TFs did not, as determined by enzyme assays and zymography. But *amyL* transcripts were synthesized in *L. brevis* TFs. In terms of plasmid stability, pSJE, a theta-type replicon, was more stable than pCW4, an RCR (rolling circle replication) plasmid, in *L. brevis* without antibiotic selection.

Key words: gene expression, α-amylase, *Lactobacillus brevis*, pCW4, pSJE

INTRODUCTION

Lactobacillus brevis is one of the major lactic acid bacteria (LAB) isolated from *kimchi* and other fermented vegetables (1,2). It produces carbon dioxide and ethanol in addition to lactic acid from carbon sources such as glucose, thus contributing significantly to the unique taste of *kimchi* (3). Although *L. brevis* is an important organism with industrial applications, few efforts have been made for strain improvement. Strain improvement via genetic engineering methods has great potential for developing strains for various commercial applications. There have been only a few reports on the heterologous gene expression in *L. brevis* as a host (4,5). For successful heterologous gene expression in a specific host, availability of suitable vectors are essential. pSJE, an *E. coli-Leuconostoc* shuttle vector, was constructed previously (6) and successfully employed for heterologous expression of *aga* encoding α-galactosidase from *Leuconostoc mesenteroides* in *Leuconostoc citreum* (7). In this communication, heterologous expression of *amyL* encoding α-amylase from *Bacillus licheniformis* in *L. brevis* 2.14 is reported. *amyL* encodes a 55-kDa α -amylase, and its gene was successfully expressed in *Lactobacillus casei* strains (8).

MATERIALS AND METHODS

Bacterial strains and culture conditions

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

Escherichia coli cells were grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation. *L. brevis* 2.14 was grown in MRS (Difco Lab, Detroit, MI, USA) broth without agitation at 37°C. Antibiotics were used at the following concentration: erythromycin (Em), 200 μg/mL for *E. coli*, 5 μg/mL for *L. brevis*.

Plasmid construction

The α-amylase gene in pTA322 originated from *Bacillus licheniformis* ATCC 27811 (9). A 3 kb fragment encompassing *amyL* was amplified by PCR and subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The fragment was then moved into the unique *Not*I site of pSJE (6), resulting in pSJETα. In the same manner, pSJ33ETα was obtained from pSJ33E (6). pCW4Tα, pCW4 (10) containing 3 kb *amyL* at its *Not*I site, was constructed previously (11).

DNA isolation and manipulation

Plasmid DNA from *E. coli* was prepared using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA), and plasmid DNA from *L. brevis* was prepared by the method of O'Sullivan and Klaenhammer (12). Restriction enzyme digestions were performed in accordance with the supplier's instructions (Promega) and DNA ligations were conducted with T4 DNA ligase (Promega) at 4°C for overnight. DNA fragments for subcloning were isolated from agarose gels using a Gel SV gel extraction kit (General Biosystem, Seoul, Korea). Agarose

†Corresponding author. E-mail: jeonghkm@gsnu.ac.kr
Phone: +82-55-751-5481, Fax: +82-55-753-4630

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Reference
Escherichia coli		
DH5α	*supE44* Δ*lac*U169(φ80*dlacZ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Gibco BRL
Lactobacillus brevis 2.14	Sak^-, Imm^-, indicator strain for sakacin A	Axelsson et al. (18)
Plasmids		
pCW4	5.3 kb, an *E. coli*-lactobacilli shuttle vector derived from pC7	Park et al. (10)
pCW4Tα	pCW4 containing *amyL* as a 3-kb *Not*I fragment from *Bacillus licheniformis*	Jeong et al. (11)
pSJE	pSJ33E with 1.16 kb *Fsp*I fragment deleted, 6.6 kb, Em^r	Jeong et al. (6)
pSJETα	pSJE containing *amyL* as a 3-kb *Not*I fragment from *Bacillus licheniformis*	This study
pSJ33E	A shuttle vector for *E. coli* and *Leuconostoc* sp, 7.7 kb, Ap^r, Em^r	Jeong et al. (6)
pSJ33ETα	pSJ33E containing *amyL* as a 3-kb *Not*I fragment from *Bacillus licheniformis*	This study
pNZ8020	a pNZ8010 derivative carrying the *gusA* gene carrying MCS2	de Ruyters et al. (19)

gel (1%, w/v) electrophoresis was conducted with Tris-Acetate-EDTA buffer (pH 8.0).

Electroporation

Transformation of *E. coli* and *L. bevis* cells was performed by the electroporation method. Frozen *E. coli* competent cell preparation and electroporation procedures were followed by the method of Dower et al. (13). Frozen competent *L. brevis* 2.14 cells were prepared by the method of Berthier et al. (14). A single colony on MRS agar plate was inoculated into 5 mL of MRS broth and incubated for 8 hr at 37°C. Then the culture was inoculated into 500 mL of MRS broth and incubated at 37°C. When the optical density (at 600 nm) reached 0.5, cells were recovered by centrifugation at $5,000 \times g$ for 10 min at 4°C, resuspended in 500 mL of 10 mM $MgCl_2$, and centrifuged for 10 min. Cells were washed with 10 mM $MgCl_2$ once more and resuspended in 200 mL of 0.5 M sucrose containing 10% glycerol. Cells were recovered by centrifugation at $5,000 \times g$ at 4°C for 15 min and washed with 200 mL of 0.5 M sucrose containing 10% glycerol once more. Cells were resuspended in 1/200 original culture volume of 0.5 M sucrose containing 10% glycerol and then stored at -76°C. Plasmid DNA was added into 40 μL of the competent cells, the mixture was transferred to a cold electroporation cuvette (0.1 cm), and a single pulse was applied (25 μF capacitance, 200 Ω resistance, and a field strength of 15 kv/cm) with GenePulserII (Bio-Rad, Hercules, CA, USA). The pulsed mixture was immediately diluted with 1 mL of MRS broth, incubated for 2 hr at 37°C, and then spread on MRS plates with Em (5 μg/mL).

Enzyme assay

Qualitative α-amylase activity measurements were done by staining plates containing soluble starch (1.0%, w/v) with 10 mM I_2-KI solution and measuring the size of clear zones. For quantitative α-amylase assays, the method described by Bernfeld (15) was used. Each strain was cultivated in MRS broth or LB until optical density at 600 nm reached 0.9 to 1.0. Then each culture was subjected to centrifugation, $5,000 \times g$ for 10 min at 4°C. Culture supernatant was decanted into a clean tube. Cell pellet was resuspended in 0.2 M sodium acetate buffer (pH 5.4), 0.2 volume of the original culture volume. The suspension was placed on ice and subjected to sonication using Bandelin Sonopuls HD60 homogenizer. Sonicated cells were centrifuged at $5,000 \times g$ for 10 min at 4°C, the supernatant was recovered as a cell pellet fraction, and used for assays. 0.5 mL of sample was mixed with 2 mL of acetate buffer (pH 5.4), 5 mL of 0.5% soluble starch, 1 mL of 1% NaCl, 0.5 mL of distilled water, and then incubated at 65°C for 30 min. The reaction was stopped by cooling on ice. The amount of released reducing sugars was measured by the dinitrosalicylic acid (DNS) method. One enzyme unit was defined as the amount of enzyme which released 1 μmol of reducing sugars (maltose) from soluble starch at 65°C per min.

SDS-PAGE and zymogram

SDS-PAGE was carried out according to the method of Laemmli (16). Stacking gel (4%) and separating gel (12%) contained 0.25% soluble starch. Enzyme samples were mixed with equal volumes of 2× sample buffer (2.3% SDS, 2.25% glycerol, 0.25% β-mercaptoethanol, 0.001% bromphenol blue and 62 mM Tris-HCl, pH 6.8) and heated for 5 min at 100°C. After electrophoresis, one half of the gel was stained with coomassie brilliant blue, and the other half was washed four times (for 30 min each) in 10 mM Tris-HCl (pH 6.8), and the gel was incubated overnight at 37°C in the same buffer. This washing step removed SDS and allowed renaturation of proteins for zymography.

Slot blot experiments

RNA from *L. brevis* 2.14 TFs was isolated by the Trisol-bead method, using 1 mL of Trisol (Invitrogen, Carlsbad, CA, USA) and 80 mg of zirconium-silica bead. Each 10 μg of RNA preparation was heated, denatured,

and applied onto a Hybond-XL nylon membrane (Amersham Bioscience, Piscataway, NJ, USA) using a slot blot system, SE646 (Amersham Pharmacia Biotech Inc., Uppsala Sweden) (7). A 3 kb fragment encompassing the *amyL* was amplified by PCR and used as the probe after being labeled with alkaline phosphatase using Amersham Alkphos Direct Labeling and Detection System (Amersham Bioscience). One hr of prehybridization and 12 hr of hybridization were performed in ULTRAhyb (Ambion, Austin, TX, USA) at 42°C.

Plasmid stability

Stability of pSJETα or pCW4Tα in *L. brevis* 2.14 was examined as follows (8,10). Actively growing *L. brevis* 2.14 TFs in MRS broth containing Em (5 μg/mL) were inoculated into fresh MRS broth without antibiotic, and incubated for 24 hr at 37°C. Cells were then inoculated again into fresh MRS broth without antibiotic. Daily subculturing in MRS broth without antibiotic was repeated up to 13 days. Each day, aliquots of culture were taken and serially diluted with 0.1 volumes of MRS broth. 0.1mL of diluted samples were spread onto MRS agar plates and plates containing Em (5 μg/mL), respectively. Plates were incubated at 37°C for 48 hr and the percentage of cells harboring plasmid was calculated as follows. Percentage of cells keeping plasmid=number of cells on MRS Em/ number of cells on MRS × 100.

RESULTS AND DISCUSSION

Transformation of *L. brevis* 2.14 with pCW4Tα and pSJETα

Several plasmids were tested for the transformation of *L. brevis* 2.14. One μg of plasmid DNA was used for each transformation and transformants (TFs) were counted after 36 hr of incubation at 37°C on MRS plates containing Em (5 μg/mL). The transformation efficiency of *L. brevis* 2.14 was 1.7×10^3 TFs per μg of pCW4 DNA, 1.5×10^3 TFs per μg of pSJE DNA, 1.1×10^3 TFs per μg of pSJ33E DNA, 4.5×10^3 TFs per μg of pNZ8020 DNA (Table 2). Transformation efficiencies of *L. brevis* 2.14 by three different plasmid vectors (pCW4, pSJ33E, and pNZ8020) were in the same range, around 10^3 TFs/μg of plasmid DNA, and quite lower than those of *E. coli* DH5α cells, which were at least 10^6 TFs per μg of plasmid DNA. Similarly low TF efficiencies are commonly observed for many LAB hosts. When plasmid DNA was prepared from TFs and digested with *Not*I, no difference in size of pCW4Tα and pSJETα was observed between plasmid preparations from *E. coli* and *L. brevis* 2.14 TFs, and the 3 kb *Not*I fragment encompassing *amyL* was generated as expected (results not shown).

Table 2. Transformation efficiencies of *L. brevis* 2.14 with various plasmids

Plasmid	Transformation frequency (per μg)	Reference
pCW4	1.7×10^3	Park et al. (10)
pCW4Tα	9.0×10^2	Jeong et al. (11)
pSJE	1.5×10^3	Jeong et al. (6)
pSJETα	1.3×10^3	This study
pSJ33E	1.1×10^3	Jeong et al. (6)
pSJ33ETα	5.9×10^2	This study
pNZ8020	4.5×10^3	de Ruyters et al. (19)

Expression of *amyL* in *E. coli* and *L. brevis* 2.14

Introduced *amyL* was expressed and endowed *E. coli* cells with α-amylase activities. When grown on LB plates containing soluble starch (1.0%, w/v), cells harboring either pSJETα or pCW4Tα produced halos but cells containing vector only (control) did not. Although culture supernatant showed higher enzyme activities than cell pellets, secreted α-amylase if any was not responsible for the halo formation since there was no difference in the enzyme activities of culture supernatants from recombinant cells and control. On the contrary, significant differences were observed for cell pellets containing cytoplasmic proteins. Cells harboring pSJETα had three times higher activities than cells harboring pSJE. Cells with pCW4Tα had nine times higher activities than cells with pCW4. *E. coli* does not have known secretion systems, which are essential for a protein to be transported into culture medium. Considering these facts, lysis of some *E. coli* cells might be responsible for the results shown in the Fig. 1. Lysis of some cells might be responsible for the fact that culture supernatant showed higher activities than cell pellet. Although the exact cause was unknown, the same results were obtained after separate measurements. *E. coli* cells with pCW4Tα showed higher α-amylase activities than those with pSJETα. Halo size of cells with pCW4Tα was also larger than those of cells with pSJETα (Fig. 1). This seemed to be caused by copy number differences between the two vectors.

L. brevis 2.14 harboring pSJETα or pCW4Tα exhibited no activities. *L. brevis* 2.14 TFs harboring pSJETα did not produce halos when grown on MRS plates containing glucose (0.25% , w/v) and 1% soluble starch (results not shown). *E. coli* cells were transformed with pSJETα prepared from *L. brevis* 2.14 TFs and they produced halos. This result indicated that structural change(s) in *amyL* was not the reason for poor expression of *amyL* in *L. brevis*. α-amylase assay using the DNS method confirmed that no active enzyme was produced in *L. brevis* 2.14 TFs although the same plasmid construct al-

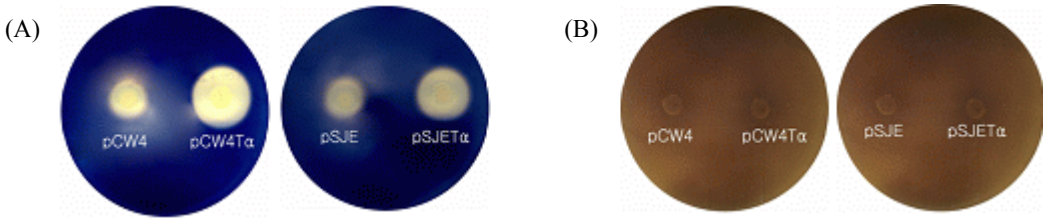

Fig. 1. Halo production by *E. coli* DH5α (A) and *L. brevis* 2.14 (B) TFs harboring pCW4Tα or pSJETα. Two μL of culture grown overnight in LB or MRS was spotted onto LB (A) and MRS plate (B) containing soluble starch (1%, w/v), respectively. Plates were incubated for 24 hr at 37°C before stained with 10 mM I_2-KI solution.

lowed synthesis of active enzyme in *E. coli* (Table 3).

SDS-PAGE and zymography for protein samples were performed as described in the materials and methods and the results are shown in Fig. 2. Although an α-amylase band could not be located on the coomassie stained gel, an active 55 kDa band was detected by zymography (Fig. 2). The size matched well with the expected size of α -amylase from *B. licheniformis* (8). No active band was detected from *L. brevis* 2.14 TFs. This result was different from the observations from *Lactobacillus casei* TFs which were constructed by introduction of pIL2530α, a pIL253 based *E. coli-Lactobacillus* shuttle vector containing *amyL* (8). Two *L. casei* TFs produced active α-amylase.

Table 3. α-amylase activities of *E. coli* and *L. brevis* TFs

Strains	Total activity (U)[1)]	
	Culture supernatant	Cell pellet
E. coli DH5α		
[pCW4]	0.0435	0.0054
[pCW4Tα]	0.0345	0.0467
[pSJE]	0.0366	0.0077
[pSJETα]	0.0484	0.0211
L. brevis 2.14		
[pCW4]	0.0437	0.0938
[pCW4Tα]	0.0423	0.1362
[pSJE]	0.0550	0.1396
[pSJETα]	0.0568	0.1674

[1)]1 U is the amount of enzyme which produces 1 μmol of reducing sugars (maltose) from soluble starch at 65°C per min.

It is unclear why active α-amylase was not produced in *L. brevis*. Low pH value of the culture media, caused by organic acids produced during lactic acid fermentation, may have inactivated α-amylase or prevented its synthesis. To examine this possibility, *L. brevis* cells harboring pSJETα were cultivated in a jar fermenter where the medium pH was constantly maintained at 7.0 to 8.0 and enzyme activities were measured, but no active enzyme was detected. Therefore, it is still an open question why active α-amylase was not produced in spite of the synthesis of *amyL* transcripts (see below).

Transcription of *amyL*

Slot blot experiments were done to see whether transcription of *amyL* occurred in *L. brevis* 2.14. Fig. 3 shows that *amyL* transcripts were synthesized in *L. brevis* 2.14 harboring either pSJETα or pCW4Tα. *L. brevis* 2.14 harboring pSJETα had stronger hybridization signal than cells harboring pCW4Tα. Future studies are necessary to determine why no active α-amylase was produced in *L. brevis* but no transcript was synthesized.

Stability of plasmids

The results of stability testing for pSJETα and pCW4Tα in *L. brevis* 2.14 are shown in Fig. 4. Without anti-

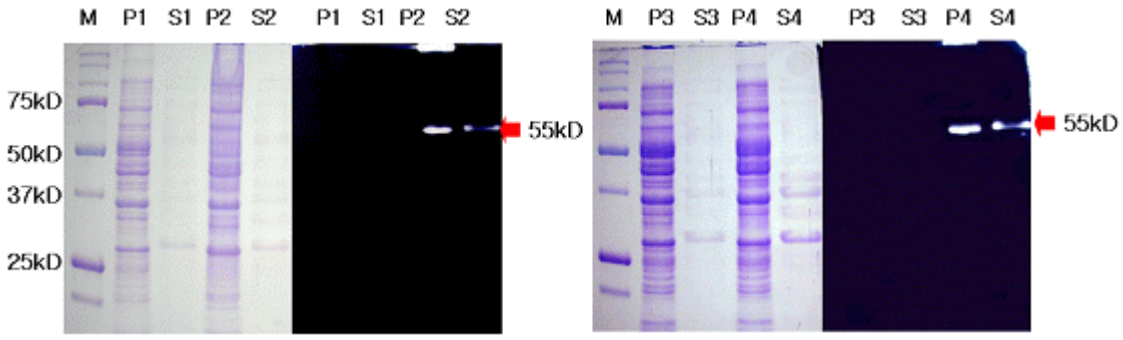

Fig. 2. SDS-PAGE and zymogram of protein samples from *E. coli* TFs. P and S indicate cell pellet and culture supernatant, respectively. M, protein size marker (Bio-Rad, Hercules, CA, USA); 1, DH5α [pCW4]; 2, DH5α [pCW4Tα]; 3, DH5α [pSJE]; 4, DH5α [pSJETα]. Arrows in the right side of zymogram indicate a 55 kDa α-amylase band.

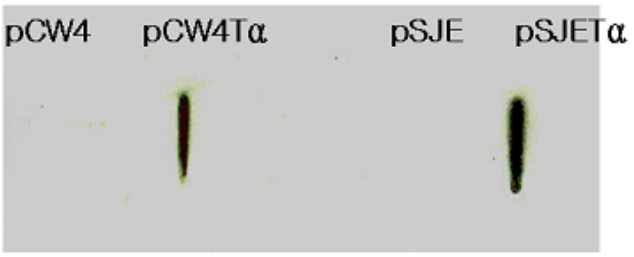

Fig. 3. Slot blot results. Ten μg of RNA samples from *L. brevis* 2.14 TFs harboring pCW4Tα and pSJETα were applied onto a membrane and 3 kb fragment containing *amyL* was labeled and used as the hybridization probe.

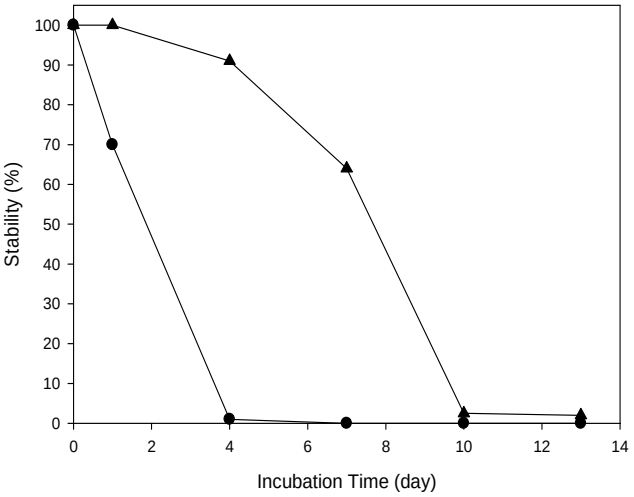

Fig. 4. Stability of pCW4Tα and pSJETα in *L. brevis* 2.14. *L. brevis* TFs were grown on MRS media without Em. One % (v/v) daily subculturing onto fresh MRS broth was continued up to two weeks and culture was taken for the presence of plasmid. ●, *L. brevis* 2.14 [pCW4Tα]; ▲, *L. brevis* 2.14 [pSJETα].

biotic selection pressure, pCW4Tα was not stable in *L. brevis* 2.14. After 4 days of daily subculturing in MRS broth, only 1.4% of cells maintained pCW4Tα and after 13 days, 0.1% of cells kept pCW4Tα. pSJETα was more stable than pCW4Tα. 3.1% of cells still maintained pSJETα after 13 days of daily subculturing in MRS broth without Em. The difference in stability reflects the different replication mode for two plasmids. pSJE replicates via theta type replication whereas pCW4 replicates via RCR, and RCR plasmids are known to become unstable if antibiotic selection pressure is missing (17). Plasmid stability is an important criterion when designing food grade vector systems since use of antibiotics is not allowed for food applications.

CONCLUSION

Heterologous *amyL* expression was tried in *L. brevis* 2.14 by introducing *amyL* using two different vectors, pSJE and pCW4. Although active α-amylase was produced in *E. coli* TFs, no active enzyme was detected from *L. brevis* 2.14 TFs. Slot blot experiment using *amyL* as a probe showed that transcription of *amyL* occurred in *L. brevis*. In *L. brevis*, pSJETα was more stable than pCW4Tα in the absence of antibiotic.

ACKNOWLEDGMENTS

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Project No. F00118, 2006). Kang Wook Lee, Ji Yeong Park, Gyoung Min Kim, Gun-Hee Kwon, Mee-Ryung Lee, and Jiyeon Chun were supported by 2nd stage of Brain Korea 21 project. Authors are grateful for the financial supports.

REFERENCES

1. Lee CW, Ko CY, Ha DM. 1992. Microfloral changes of the lactic acid bacteria during *Kimchi* fermentation and identification of the isolates. *Kor J Appl Microbiol Biotechnol* 20: 102-109.
2. Plengvidhya V, Breidt F Jr, Lu Z, Fleming HP. 2007. DNA fingerprinting of lactic acid bacteria in sauerkraut fermentations. *Appl Environ Microbiol* 73: 7697-7702.
3. Lee KS, Shin YS, Lee CH. 1998. Acid tolerance of *Lactobacillus brevis* isolated from *Kimchi*. *Kor J Food Sci Technol* 30: 1399-1403.
4. Liu S, Dien BS, Nichols NN, Bischoff KM, Hughes SR, Cotta MA. 2007. Coexpression of pyruvate decarboxylase and alcohol dehydrogenase genes in *Lactobacillus brevis*. *FEMS Microbiol Lett* 274: 291-297.
5. Iijima K, Suzuki K, Ozaki K, Yamashita H. 2006. *horC* confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45cc. *J Appl Microbiol* 100: 1282-1288.
6. Jeong SJ, Park JY, Lee HJ, Kim JH. 2007. Characterization of pFMBL1, a small cryptic plasmid isolated from *Leuconostoc mesenteroides* SY2. *Plasmid* 57: 314-323.
7. Park JY, Jeong SJ, Lee AR, Park JY, Jeong WJ, Kim JH. 2007. Expression of α-galactosidase gene from *Leuconostoc mesenteroides* SY1 in *Leuconostoc citreum*. *J Microbiol Biotechnol* 17: 2081-2084.
8. Kim JH, Woo SH. 1995. Expression of *Bacillus licheniformis* α-amylase gene in *Lactobacillus casei* strains. *J Microbiol Biotechnol* 5: 257-263.
9. Kim IC, Jang SY, Cha JH, Ko YH, Park KH, Rho HM. 1998. Cloning and expression of thermostable α-amylase gene in *Escherichia coli* from *Bacillus licheniformis* ATCC 27811. *Kor J Appl Microbiol Bioeng* 16: 369-373.
10. Park WJ, Lee KH, Lee JM, Lee HJ, Kim JH, Lee JH, Chang HC, Chung DK. 2004. Characterization of pC7 from *Lactobacillus paraplantarum* C7 derived from kimchi and development of lactic acid bacteria-*Escherichia coli* shuttle vector. *Plasmid* 52: 84-88.
11. Jeong SJ, Park JY, Kim JH, Kim GM, Chun J, Lee JH, Chung DK, Kim JH. 2006. Transformation of *Leuconostoc mesenteroides* SY1, a strain isolated from Kimchi. *J Microbiol Biotechnol* 16: 149-152.
12. O'Sullivan DJ, Klaenhammer TR. 1993. Rapid mini-prep

isolation of high quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl Environ Microbiol* 59: 2730-2733.
13. Dower WJ, Miller JF, Ragsdale CW. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* 16: 6127-6145.
14. Berthier F, Zagorec M, Champomier-Verges M, Ehrlich SD, Morel-Devile F. 1996. Efficient transformation of *Lactobacillus sake* by electroporation. *Microbiology* (Reading) 142: 1273-1279.
15. Bernfeld P. 1955. Amylases. In *Methods in Enzymology*. Academic Press, CA. Vol 1, p 149-151.
16. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
17. Kiewiet R, Seegers JF, Venema G, Bron S. 1993. The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. *Appl Environ Microbiol* 48: 726-731.
18. Axelsson A, Holck A, Birkrland SE, Aukrust T, Blom H. 1993. Cloning and nucleotide sequence of a gene from *Lactobacillus sake* Lb706 necessary for sakacin A production and immunity. *Appl Environ Microbiol* 59: 2868-2875.
19. de Ruyters PG, Kuipers OP, De Vos WM. 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* 62: 3662-3667.

(Received May 14, 2008; Accepted September 18, 2008)