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Transformation of *Leuconostoc mesenteroides* SY1, a Strain Isolated from Kimchi

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Abstract *Leuconostoc mesenteroides* SY1, a strain isolated from cabbage Kimchi, was transformed with pCW4, a shuttle vector based on a cryptic plasmid from *Lactobacillus paraplantarum* C7. α-Amylase gene, *amyL*, from *Bacillus licheniformis* was cloned into pCW4, resulting in pCW4Tα, and pCW4Tα was introduced into SY1 by electroporation. Transformation efficiency was 10^2 cells/μg plasmid DNA. *L. mesenteroides* cells harboring pCW4Tα did not show amylase activity, although *amyL* transcript was synthesized as determined by slot blot experiment. pCW4Tα was stably maintained in SY1 in the presence of erythromycin (Em, 5 μg/ml) but rapidly lost when Em was omitted. Less than 1% of the cells maintained pCW4Tα after 5 days at 30°C.

Key words: Electroporation, Kimchi LAB, heterologous gene expression, plasmid stability

The amount of commercially manufactured Kimchi at Kimchi factories has been increasing continuously along with that of internationally exported and imported Kimchi. This trend necessitates extensive studies on various factors that determine the eventual quality of Kimchi. Similar to many other fermented foods such as cheese and yogurt, the quality of Kimchi is largely determined by lactic acid fermentation carried out by lactic acid bacteria (LAB). Thus, it is important to select LAB with desirable characteristics for Kimchi fermentation or improve LAB. Considering the use of selected starter strains for Kimchi production at Kimchi factories, efforts for strain improvements become especially important [8, 9]. *Leuconostoc mesenteroides* is considered as the most important beneficial organism for Kimchi ripening; nevertheless, not much is known about this organism. No attempts to genetically improve or modify this organism have been reported for strains isolated from Kimchi, except for a report on the biochemical characterization of an acid resistant mutant [7], and no reports are available on the transformation of *L. mesenteroides* strains isolated from Kimchi. Establishing an efficient transformation procedure is the first step toward genetic engineering of an organism. In this note, we report the transformation of *L. mesenteroides* SY1, a strain isolated from Kimchi, and expression of an α-amylase gene (*amyL*) from *Bacillus licheniformis* in *L. mesenteroides* SY1. As far as we are aware, this is the first report on the transformation and heterologous gene expression in an organism isolated from Kimchi, which plays an important role during Kimchi fermentation.

L. mesenteroides SY1 was isolated from cabbage Kimchi purchased at a local market. It was identified biochemically using an API 50 kit (Biomeriux, Etoile, France) and further confirmed by 16S rDNA sequence comparison [6]. *L. mesenteroides* SY1 was grown on MRS (Difco Lab, Detroit, MI, U.S.A.) media at 30°C without agitation. *E. coli* strains were grown on LB at 37°C with vigorous shaking. *amyL* in pTA322 originated from *Bacillus licheniformis* ATCC 27811 [4]. Antibiotics were included at the following concentrations: Em, 5 μg/ml for *L. mesenteroides*, 200 μg for *E. coli*; Ap (ampicillin), 100 μg/ml for *E. coli*. pCW4Tα was obtained by inserting a 3-kb fragment encompassing the complete *amyL* from *B. licheniformis* into pCW4 [12]. The *amyL* gene was amplified by PCR using the primer pairs 5'-**CGAGCTC**CTCATGTTTGACAGCTTATC-3' and 5'-**CGAGCTC**AACTGTGATAAACTA CCGCA-3'. A *Sac*I site (bold) was introduced into each primer. The amplified 3-kb fragment was subcloned into pGEM-T Easy vector (Promega, Madison, WI, U.S.A.), and the resulting plasmid, pTATα, was introduced into *E. coli* DH5α. Then, the *amyL*

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gene from pTATα was subcloned into the unique *Not*I site of pCW4, resulting in pCW4Tα.

Frozen competent *L. mesenteroides* SY1 cells were prepared by the method modified from the procedure of Berthier *et al.* [1]. All solutions, centrifuge rotor, tubes, and e-tubes were prechilled, and care was exercised to maintain cells at refrigerating temperature during the procedure. A single colony on MRS plate was inoculated into 30 ml of MRS broth and incubated overnight at 30°C. Two and a half ml of overnight culture was inoculated into 250 ml of MRS broth (1% inoculum) containing 40 mM DL-threonine and incubated at 30°C until OD_{600} reached 0.5. The culture was rapidly cooled in an ice-water bath for 10 min, and cells were recovered by centrifugation (7,000 ×*g*, 10 min) at 4°C. Cells were resuspended in 200 ml of 10 mM $MgCl_2$ and centrifuged again. Cell washing with 10 mM $MgCl_2$ was repeated once more, and cells were resuspended in 150 ml of 10% glycerol/0.5 M sucrose. Cells were recovered by centrifugation (7,000 ×*g*, 15 min) at 4°C, and washing with 10% glycerol/0.5 M sucrose was repeated again. Cells were resuspended in 0.5 ml of 10% glycerol/0.5 M sucrose, and 50 μl was taken out to check whether an arc had occurred during electroporation. If no arc was generated, 50 μl aliquots were dispensed into prechilled e-tubes, and the tubes were strored at −76°C. Just before the transformation experiment, cells were thawed on ice. Plasmid DNA was added and cells were stood on ice for 3 min. GenePulser II (Bio-Rad, Hercules, CA, U.S.A.) was used, and a single pulse was applied (0.1 cm cuvette, 25 μF capacitance, 200 Ω resistance, and a field strength of 1.8 kv/cm). The pulsed mixture was immediately diluted with 1 ml of MRS broth containing 80 mM $MgCl_2$, incubated for 2 h at 30°C, and then 0.1 ml aliquots were spread onto MRS plates containing 1% soluble starch and Em (5 μg/ml). *E. coli* competent cell preparation and electroporation procedures were carried out by the method of Dower *et al.* [2]. Plasmid DNA from *E. coli* was prepared using a QIAprep kit (Qiagen, Valencia, CA, U.S.A.), and plasmid preparation from *L. mesenteroides* was done according to the method of O'Sullivan and Klaenhammer [10]. About 100 transformants were obtained for 1 μg of pCW4Tα after 24 h of incubation at 30°C. The efficiency was much lower than those reported for *Lactococcus lactis* strains (10^6 transformants/μg DNA); however, this low efficiency is commonly observed for LAB isolated from foods and other natural environments. The low efficiency is obviously a stumbling block that must be solved for the genetic engineering of LAB. Figure 2 shows the plasmid prepared from *E. coli* and *L. mesenteroides* transformants. No apparent size difference was observed between DNA from *E. coli* and that from *L. mesenteroides*. The quality of pCW4Tα from *L. mesenteroides* SY1 was not good, compared with that from *E. coli*, resulting in incomplete digestion by *Nco*I.

L. mesenteroides SY1 transformants harboring pCW4Tα did not produce halos around colonies on MRS (0.25%

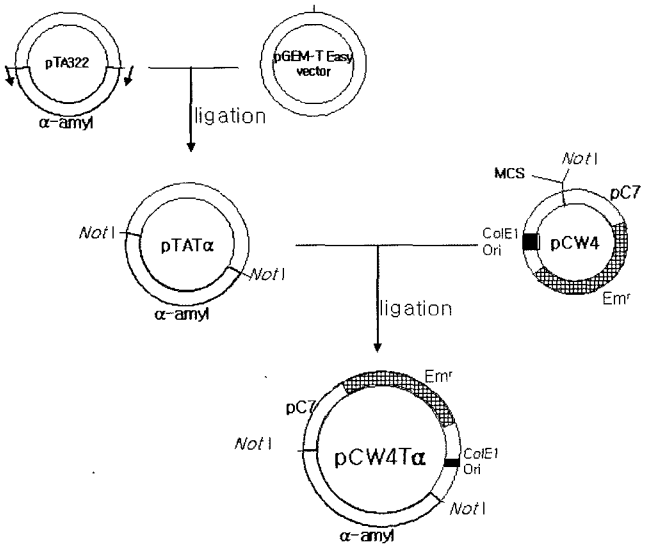

Fig. 1. Construction of pCW4Tα.
The α-amylase gene was amplified from pTA322 and ligated to pGEM-T Easy vector to yield pTATα. Then, the α-amylase gene of pTATα was subcloned into the unique *Not*I site of pCW4.

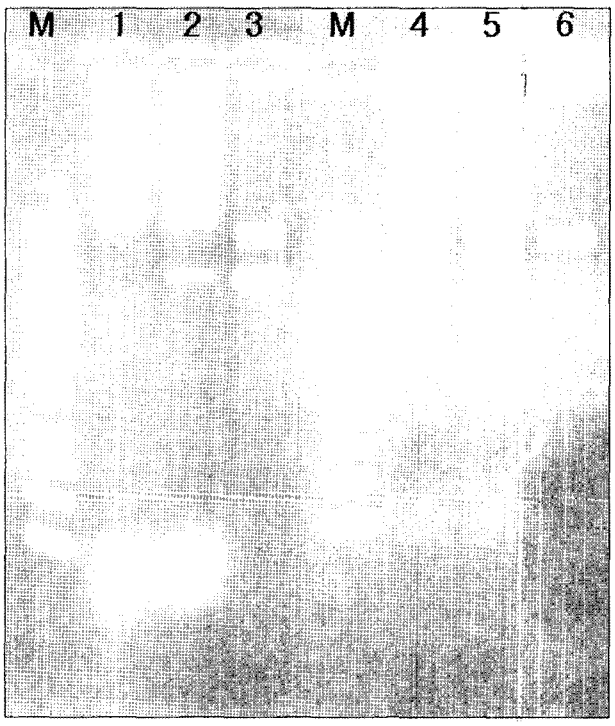

Fig. 2. Agarose gel electrophoresis of plasmid prepared from DH5α transformed with pCW4Tα derived from *L. mesenteroides* SY1 transformant.
M, 1 kb ladder size maker (BRL); 1, plasmid profile of *L. mesenteroides* SY1; 2, pCW4Tα prepared from *L. mesenteroides* SY1 transformant; 3, pCW4Tα prepared from *E. coli* DH5α transformant; 4, *Nco*I-digested DNA from lane 1; 5, *Nco*I-digested DNA from lane 2; 6, *Nco*I-digested DNA from lane 3.

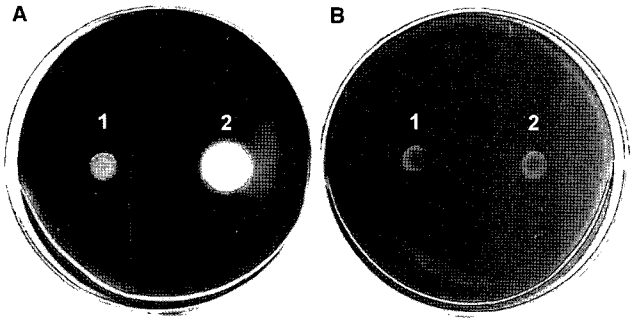

Fig. 3. Halo production by *E. coli* DH5α and *L. mesenteroides* SY1 transformant harboring pCW4Tα.
A. LB soluble starch plate stained with KI/I_2. B. MRS soluble starch plate (glucose 0.25%, w/v) stained with KI/I_2 1, *E. coli* (A) or *L. mesenteroides* (B) cells with pCW4; 2, cells with pCW4Tα.

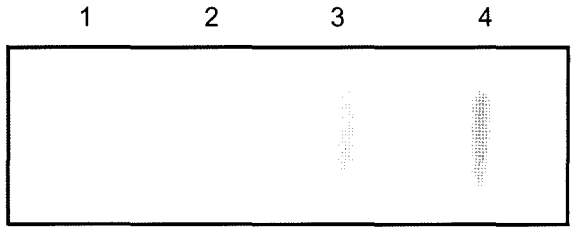

Fig. 4. Quantification of the *amyL* transcript from *L. mesenteroides* SY1.
Ten μg of RNA was spotted onto a Hybond-XL nylon membrane (Amersham Biosciences) and hybridized with a 3-kb radiolabeled *amyL* probe. 1, SY1 [pCW4] grown on 2% glucose-containing MRS medium; 2, SY1 [pCW4Tα] grown on 2% glucose-containing MRS medium; 3, SY1 [pCW4Tα] grown on 1% gluose-containing MRS medium; 4, SY1 [pCW4Tα] grown on 0.25% glucose-containing MRS medium.

glucose, w/v) plates containing soluble starch (1%, w/v) (Fig. 3b), although *E. coli* transformants with pCW4Tα produced halos. *E. coli* cells transformed with pCW4Tα, which was obtained from the *L. mesenteroides* SY1 transformant, also produced halos, indicating that pCW4Tα was not modified in SY1. The α-amylase assay confirmed that no active enzyme was produced in SY1, although the same plasmid construct produced active enzyme in *E. coli*. SY1 transformants grown in a jar fermenter, where the medium pH was constantly maintained at 7.0, but also did not show any significant activity, either (data not shown).

Slot blot experiments were done to see whether *amyL* transcription occurred. Total RNA from *L. mesenteroides* SY1 harboring pCW4Tα was isolated using a FastRNA Pro Blue kit (Q-Bio Gene, Carlsbad, CA, U.S.A.). Ten μg each of preparation was heated, chemically denatured, and applied onto a Hybond-XL nylon membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.) by a slot blot system (SE646, Amersham Pharmacia Biotech Inc., U.S.A.) [13]. A 300 bp (base pair) fragment, corresponding to the internal region of *amyl*, was amplified by PCR and used as a probe after being labeled with ^{32}P-CTP by the Rediprime II DNA labeling System (Amersham). Prehybridization (1 h) and hybridization (overnight) were performed in ULTRAhyb (ultrasensitive hybridization buffer, Ambion, Austin, TX, U.S.A.) at 42°C, and two posthybridization washes were carried out at 42°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)-0.1% sodium dodecyl sulfate prior to film exposure (Hyper film ML). Figure 4 shows that the *amyL* transcript was synthesized in the SY1 transformant that did not show any enzyme activity. SY1 cells grown on MRS broth containing 0.25% glucose produced the strongest hybridization signal, and cells grown on 2% glucose had the weakest signal. SY1 cells with pCW4 did not produce any signal, indicating that the *amyL* transcript was synthesized in SY1 and its synthesis was under catabolite repression. SDS-PAGE showed that *E. coli* [pCW4Tα] produced a 55 kDa α-amylase band, which was missing in cells harboring pCW4 only. However, no such band was observed in protein extract from SY1 [pCW4Tα] (data not shown). The reason why SY1 [pCW4Tα] failed to produce active α-amylase is not clear, but one possible explanation is that the amino acid sequence of amylase might not be ideal for translation in *L. mesenteroides*. α-Amylase has 512 aa (amino acids), and its signal peptide consists of 29 aa. Six alanines are found in the signal peptide and five are present in a row (26–30 aa) constituting the cleavage site. Alanine is the most abundant amino acid, which appears 41 times, constituting 8% of total amino acids. When the codons of six alanines in the signal peptide were examined, GCG appeared three times, and this codon was rare in *L. mesenteroides* (http://www.kazusa.or.jp/codon/). Thus, the high content of alanine in α-amylase might be the reason for poor translation. More detailed studies are necessary to find the optimum conditions for heterologous gene expression in SY1. It is noteworthy that the same *amyL* gene has successfully been expressed in *L. casei* strains [5]; therefore, the result in this work emphasizes the fact that successful expression of a heterologous gene in Kimchi LAB depends on careful selection of the target gene-host organism.

Stability of pCW4Tα in *L. mesenteroides* SY1 was examined as follows [5, 11]. Actively growing cells containing pCW4Tα in MRSEm (5 μg/ml) broth were 1% (v/v) inoculated into fresh MRS broth without antibiotic and incubated for 24 h at 30°C. Cells were then inoculated again into fresh MRS medium without antibiotic. Daily subculturing into MRS broth without antibiotic was repeated up to one week. Each day, aliquots of culture were taken and serially diluted with 0.1× MRS broth. Diluted samples (0.1 ml) were spread onto MRSEm (5 μg/ml) or MRS plates, and incubated at 30°C for 48 h. The percentage of cells harboring pCW4Tα was calculated by dividing the number of cells on MRSEm plates by the number of cells on MRS plates and multiplying by 100. The results are shown in Table 1. Without Em, pCW4Tα was not stable

Table 1. Stability of pCW4Tα in *L. mesenteroides* SY1.

Incubation time (days)							
0	1	2	3	4	5	6	7
100%	54.9	16.3	8.1	2.2	0.7	0.4	0.1

in *L. mesenteroides* SY1, and only 0.1% of cells still maintained pCW4Tα after 1 week. Usually, RCR (rolling circle replication) plasmids such as pCW4 are unstable without antibiotic selection [3]. pIL2530α, a theta-type plasmid and containing the same *B. licheniformis* α-amylase gene, was more stable in *Lactobacillus casei* ATCC 4646. It took 3 weeks to reach 0.1% [5].

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