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Transformation of silage-making *Lactobacillus* strains by electroporation with plasmid vectors

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Several newly isolated strains of *Lactobacillus* for silage fermentation were transformed with plasmid vectors, pGK12 and pSA3, by electroporation. Transformation efficiency depended on the electric field strength, time-constant, polyethyleneglycol (PEG) molecular weight in electroporation buffer and the incubation temperature after exposition of pulses. Transformation of *L. pentosus* NGRI0225 resulted in the maximum transformation efficiency of 5.7×10^5 transformants per μg DNA of pGK12 with an electric field strength of 7.0 kV/cm, time-constant of 6.2 ms and 30% (W/V) of PEG 1000. Plasmid DNAs isolated from all transformants did not show any detectable rearrangements or deletions under these conditions.

INTRODUCTION

Lactobacillus species are gram-positive bacteria, which are used as inocula of grass silage, meat and lactic acid beverages. In spite of their importance, very little is known about their efficient transformation systems. Polyethylene glycol (PEG)-induced protoplast transformation (Leer *et al.*, 1987; Badii *et al.* 1989) and transfection (Cosby *et al.*, 1988) of Lactobacilli are possible but are not very efficient.

Electroporation is a relatively new method for bacterial transformation that is rapidly gaining acceptance. This method has opened the way to genetic analysis and manipulations for many kinds of bacteria. Electroporation for Lactobacilli has also been described (Aukrust and Nes 1988; Luchansky *et al.*, 1988; Josson *et al.*, 1989; Badii *et al.*, 1989; Bringel *et al.*, 1989), but the transformation efficiencies obtained were generally very low. The optimal method of transformation (5×10^6 transformants/μg plasmid DNA) has been obtained by Bringel *et al.* (1990).

We attempted to employ the newly isolated strains of *Lactobacillus* for silage fermentation, and systematically investigated the effects of several factors on the transformation efficiency. This paper describes the optimal transformation of these *Lactobacillus* strains by electroporation with plasmids, pGK12 and pSA3.

MATERIALS AND METHODS

Bacterial strains and plasmids

Lactobacillus strains used in this study were *L. plantarum* NGRI0315, *L.*

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plantarum NGRI0529, *L. pentosus* NGRI0225, *L. pentosus* NGRI0524, *L. rhamnosus* NGRI0110 and *L. plantarum* IFO3070. Strains NGRI were isolated from grass silages gathered from south-west Japan (Tanaka *et al.*, 1994; 1998; Doi *et al.*, 1997). These strains were grown in MRS (Difco) broth at 37 °C. The MRS agar medium containing antibiotics erythromycin and lincomycin was also used as the selection medium for transformants.

Plasmids pSA3 (10.2 kbp, Em^r; Dao *et al.*, 1988) and pGK12 (4.4 kbp, Em^r; Kok *et al.*, 1984) were kindly provided by Dao M. L (University of Oklahoma Health Sciences Center, U. S. A) and Kok J (University of Groningen, Netherlands), respectively.

Electroporation protocol.

Electroporation was done by using a BTX transformation system ECM600 (San Diego, Calif, USA). A pulse produced by discharge of a capacitor has an exponential decay waveform. Time-constant depends on the total resistance (R, in Ω)-capacitance (C, in F) of the system: $\tau = R \times C$. Therefore, τ describes the shape of the decay waveform and is the time required for the electric field strength (V/cm) to decline to 1/e(36.8%) of the initial value.

To obtain competent cells, the cells were cultured in MRS broth at 37 °C for 12 hr, and then transferred to MRS containing 1% (w/v) of glycine and grown at 32 °C for 2 hr, until O.D. at 600 nm of 0.4-0.6. The cells were harvested by centrifugation at 4 °C, 6000 rpm. After the cells were washed with bidistilled water sterilized by autoclaving, the pellet was resuspended in a solution of 30% (w/v) PEG and then aliquots were stored at -85 °C until use.

A part (100 μl) of the cell suspension was thawed on ice and mixed with 1 μl of plasmid DNA dissolved in TE buffer. The mixed suspensions were transferred to ice-cooled electroporation cuvettes (2-mm electrode gap) and then exposed to a single electrical pulse. The cuvettes were then placed on ice for 10 min, after which they were transferred to MRS medium and incubated at 32 °C or 37 °C for 4 hr to allow expression of erythromycin-resistant gene, and they were then spreaded on MRS agar medium containing 5 μg of erythromycin (Sigma) per ml and 10 μg of lincomycin (Sigma) per ml for incubation at 32 °C for 2 days.

Isolation and analysis of plasmids DNA

Erythromycin-resistant transformants were grown at 37 °C for 12 hr in MRS medium containing 10 μg of etythromycin per ml. Plasmids were isolated as described elsewhere (Anderson and McKay 1983) and analyzed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

Effect of electric field strength

The electric field used for electroporation is important and the optimal electric field depends on the bacterial species and strains.

To determine the optimal electric field for electroporation, we exposed the cells to single decayed pulses with electric field strength from 5 kV/cm to 8 kV/cm when capacitance was fixed at 25 μF. As shown in Fig. 1, the maximum transformation

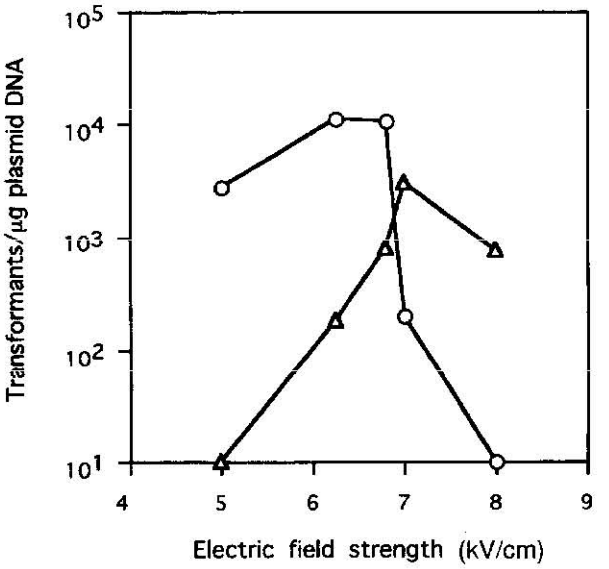

Fig. 1. Effect of electric field strength on transformation efficiency of *Lactobacillus plantarum* NGRI0315 and *Lactobacillus pentosus* NGRI0225 with plasmid pGK12. The time-constant was fixed at 6.2 ms.

Symbols: ○ : *Lactobacillus plantarum* NGRI0315
△ : *Lactobacillus pentosus* NGRI0225

efficiency was obtained for *L. plantarum* NGRI0315 and *L. pentosus* NGRI0225: the former was 1.1×10^4 transformants per μg pGK12 and the latter was 3.0×10^3 transformants per μg pGK12 at 6.25 kV/cm, 7.0 kV/cm, respectively. Transformation efficiency with electric field strength of 8 kV/cm decreased because of the irreversible death of cells caused by higher electric pulsation (Chassy and Flickinger 1987). The amplitude of the electric field therefore had an effect on the efficiency of electroporation. There was a difference in optimal electric field between *L. plantarum* NGRI0315 and *L. pentosus* NGRI0225. This result indicated that the optimal electric field was different for each strain.

Effect of time-constant

The optimal time-constant was examined for the resistance range of between 186 Ω and 720 Ω when capacitance was fixed at 25 μF, resulting in time-constants of 4.6 to 18 ms. Maximum transformation efficiency was obtained with 6.2 ms for *L. pentosus* NGRI0225 and *L. planatarum* NGRI0315, as shown in Fig. 2. The time-constant had a common effect in both strains. The transformation efficiency was decreased gradually over the optimum time-constant, 6.2 ms. This might be due to cell death or DNA damage occurring at higher time-constant (Bringel *et al.*, 1990).

Effect of PEG molecular weight in electroporation buffer

Effect of PEG molecular weight in electroporation buffer on transformation efficiency was also investigated. PEG molecular weight was varied from 1000 to 6000 when its concentration was held at 30% (w/v). As shown in Fig. 3, the optimal PEG molecular

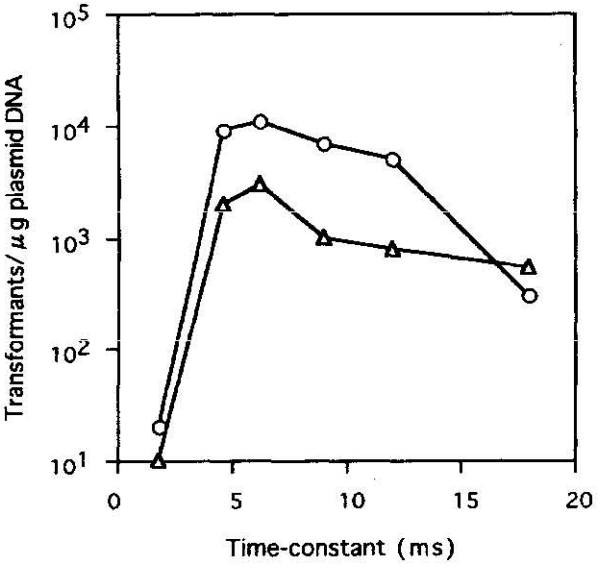

Fig. 2. Effect of time-constant on transformation efficiency of *Lactobacillus* strains NGRI0225 and NGRI0315 with plasmid pGK12. The electric field strength of *Lactobacillus* strains NGRI0225 and NGRI0315 were 7.0 kV/cm and 6.25 kV/cm, respectively, and capacitance was fixed at 25 μF.

Symbols: ○ : *Lactobacillus plantarum* NGRI0315
△ : *Lactobacillus pentosus* NGRI0225

weight was obtained with PEG 1000 and transformation efficiency of *L. plantarum* NGRI0315 was 3.6×10^4 transformants/μg pGK12 DNA. Transformation efficiency distinctly decreased with increasing PEG molecular weight, PEG 6000 had no effect. Similar observations were reported in *Lactobacillus hilgardii* (Josson *et al.*, 1989), *Bacillus thuringiensis* (Mahillon *et al.*, 1989) and *Streptomyces rimosus* R6 (Pigac *et al.*, 1995). The positive effect of PEG would be attributed to volume exclusion, interactions with cell membranes, and the increased survival of electroporated cells.

Effect of incubation temperature after exposition of pulse

Effect of incubation temperature after exposition of pulse was also estimated. After the cells were exposed to a single pulse, they were incubated for 4 hr to allow for expressions of erythromycin-resistant gene. the cultures were incubated at 32 °C or

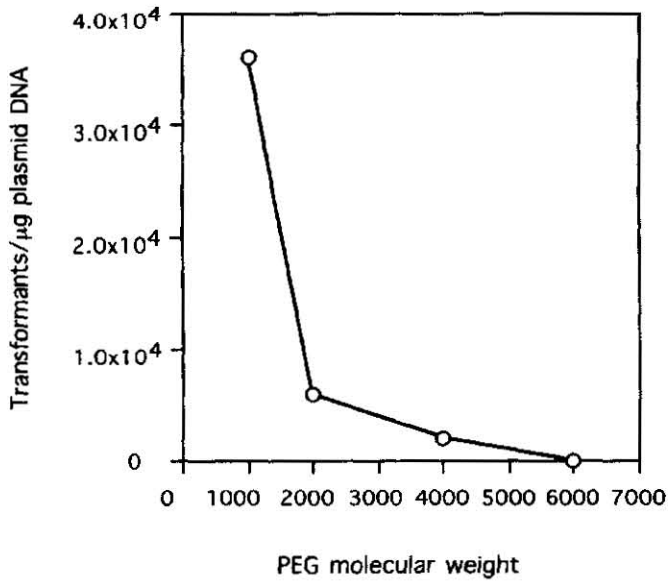

Fig. 3. Effect of PEG molecular weight on transformation efficiency of NGRI0315 with plasmid pGK12. PEG concentration was kept at 30% (W/V).

Table 1. Effect of incubation temperature after exposition of a pulse on transformation efficiency of *Lactobacillus pentosus* NGRI0225 and *Lactobacillus plantarum* NGRI0315 with plasmids pGK12 and pSA3.

Strains	Plasmids	Transformants/μg plasmid DNA	
		32 °C	37 °C
NGRI0315	pGK12	2.8×10^{3}	1.1×10^{4}
	pSA3	4.2×10^{3}	0–10
NGRI0225	pGK12	1.2×10^{3}	3.0×10^{3}
	pSA3	5.5×10^{3}	0–20

37 °C, the results are shown in Table 1. When the cells were incubated at 32 °C, the transformation efficiencies by both plasmids pGK12 and pSA3 were high (2.8×10^{3}–5.5×10^{3} transformants/μg plasmid DNA). However when the cells were incubated at 37 °C, the transformation efficiency was high only in using the pGK12, but transformants were hardly obtained when using the pSA3. These results indicated that the transformation efficiencies depended on incubation temperature after exposition of a pulse and type of plasmids.

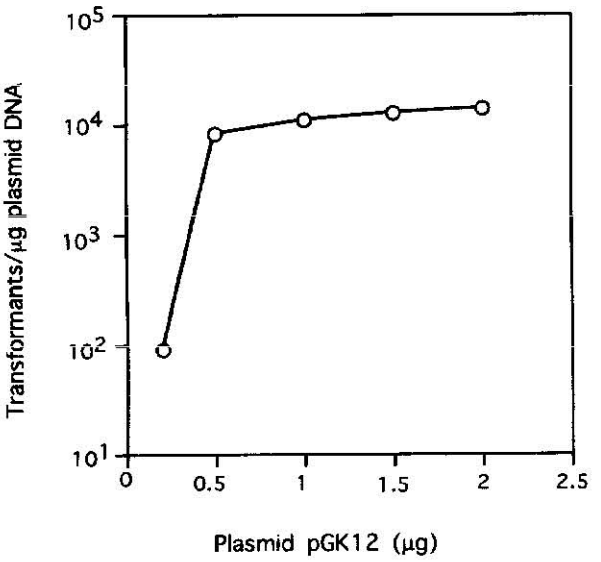

Fig. 4. Effect of DNA concentration on transformation efficiency of *Lactobacillus plantarum* NGRI0315 with plasmid pGK12. Plasmid DNA was added to the cells suspended in electroporation–buffer. A 100 μl of suspension was exposed to single pulse of electric field strength of 6.25 kV/cm. The capacitance was fixed at 25 μF.

Effect of plasmid DNA concentration

Transformation efficiency using electroporation was highly dependent on DNA concentration. As shown in Fig. 4, there was a linear relationship between transformation efficiency and DNA concentration ranging from 0.2 to 0.5 μg/ml of pGK12 DNA. However, transformation efficiency was hardly increased when DNA concentration was more than 0.5 μg/ml. Similar results were obtained by Bringel *et al.* (Bringel *et al.*, 1990)

Plasmids analysis of transformants

Plasmids from various transformants were analyzed by agarose gel electrophoresis. As shown in Fig. 5, all transformants analyzed had plasmid DNAs with the same mobilities as those of the authentic plasmid DNAs. The restriction patterns of plasmid DNAs using *Eco*RV and *Hin*dIII were also identical in all transformants (data not shown). The results indicated that the plasmid DNAs in the transformants were replicated exactly and did not suffer any significant modifications such as rearrangements or deletions.

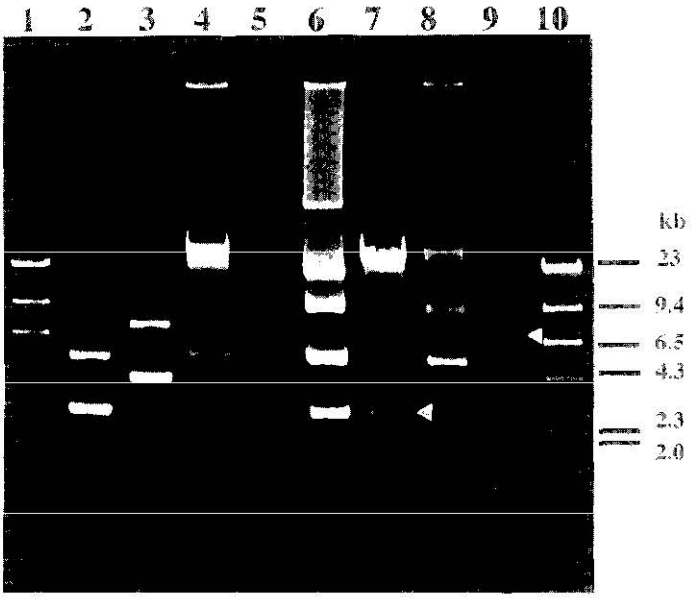

Fig. 5. Agarose gel electrophoresis analyses of plasmids from *Lactobacillus plantarum* NGRI0315, *Lactobacillus pentosus* NGRI0225 and *E. coli.* HB101. Lane 1 and 10, *Hin*dIII-digested λ DNA; Lane 2, pGK12 (HB101); Lane 3, pSA3 (HB101); Lane 4, Wild type NGRI0315; Lane 5, Wild type NGRI0225; Lane 6, pGK12 (NGRI0315); Lane 7, pGK12 (NGRI0225); Lane 8, pSA3 (NGRI0315); Lane 9, pSA3 (NGRI0225).

Table 2. Transformation efficiency of several silage-making *Lactobacillus* strains with plasmids pGK12 and pSA3.

Strains	Transformants/μg of plasmid pGK12	Transformants/μg of plasmid pSA3
Lactobacillus plantarum NGRI 0315	1.1×10^{5}	4.2×10^{3}
Lactobacillus plantarum NGRI 0529	2.2×10^{5}	3.3×10^{2}
Lactobacillus pentosus NGRI 0225	3.0×10^{3} (5.7×10^{6})*	5.4×10^{3}
Lactobacillus pentosus NGRI 0524	3.1×10^{6}	3.9×10^{5}
Lactobacillus rhamnosus NGRI 0110	1.0×10^{5}	1.2×10^{2}
Lactobacillus plantarum IFO 3070	1.0×10^{5}	1.0×10^{2}

*, Plasmid pGK12 used was isolated from a transformant of *L. pentosus* NGRI0225

Application to other *Lactobacillus* strains

We attempted to apply the above condition of electroporation obtained for *Lactobacillus* strains NRGI0225 and NGRI0315, to several other silage-making *Lactobacillus* strains. As shown in Table 2, *L. rhamnosus* NGRI0110, *L. plantarum* 3070, *L. pentosus* NRGI0524 and *L. plantarum* NGRI0529 were able be transformed with pGK12 and pSA3 at various transformation effeciencies. Their transformation efficiencies ranged from 1.0×10^2 to 3.1×10^3 transformants per μg plasmid DNA. In order to exclude restriction modification (R/M) systems in *E. coli*, transformation was carried out with plasmid pGK12 isolated from a transformant of *L. pentosus* NGRI0225. As shown Table 2, datum in parenthesis of higher transformation efficiency was obtained with 5.7×10^5 transformants per μg plasmid DNA.

These results demonstrated that plasmid DNA could be efficiently introduced into silage-making *Lactobacillus* strains by electroporation. This technique is simple, rapid and applicable to many kinds of silage-making *Lactobacillus* strains. It will become a useful technique for further research in the molecular genetics of silage-making *Lactobacillus* strains

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