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Glass bead-based transformation method for lactic acid bacteria

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ABSTRACT: Electroporation has been considered as a popular transformation method for lactic acid bacteria. However, it is inaccessible to some laboratories because of its requirement of expensive and specialized equipment. In this study, we propose a transformation method for lactic acid bacteria which does not require equipment other than that readily available in a typical laboratory. This method is based on agitation of mutanolysin-treated bacterial cells with glass beads in the presence of plasmid DNA and polyethylene glycol. By using the basic protocol of glass bead transformation, *Leuconostoc dextranicum* ATCC 19255 was successfully transformed with pGK12 at the frequency of 323 per µg of plasmid DNA. Effects of many parameters on the transformation frequency were studied to optimize the transformation protocol. By using the optimized protocol of glass bead transformation, introduction of pGK12 into mutanolysin-treated cells of several strains of lactic acid bacteria was achieved with high reproducibility and acceptable efficiency.

KEYWORDS: polyethylene glycol, mutanolysin

INTRODUCTION

Lactic acid bacteria are Gram-positive, non-sporulating microaerophilic bacteria. They include species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*[1]. Many of them have been used as starter cultures for the fermentation of foods and beverages, where they contribute to the development flavour and aroma, and retard spoilage[2]. They have also been considered putative probiotic microorganisms[3]. Lactic acid bacteria have been widely accepted as useful. Genetic studies of these organisms, however, are still limited, in part due to the lack of a simple, inexpensive, and reliable transformation procedure. The thick peptidoglycan layer present in lactic acid bacteria cell walls is considered a potential barrier to DNA uptake, preventing transformation of these organisms from occurring naturally, but it can be accomplished by particular transformation methods. Protoplast transformation and electroporation have been successfully employed to introduce DNA from the surrounding environment into lactic acid bacteria.

Protoplast transformation was developed for enzymatic removal of the cell wall to create protoplasts. In the presence of polyethylene glycol, DNA uptake by protoplasts is facilitated. If maintained under osmotically stabilized conditions, transformed protoplasts regenerate their cell walls and express the transformed DNA. Protoplast transformation has been developed for some lactic acid bacteria[4–7]. However, the method is tedious and time-consuming, and parameters must be optimized for each strain. Moreover, transformation efficiencies are often low and highly variable.

Protoplast transformation has become less popular since the advent of electroporation, a technique involving the application of a high voltage electric pulse of short duration to induce the formation of transient pores in cell walls and membranes. Under appropriate conditions, DNA present in the surrounding environment may enter through the pores. Electroporation overcomes the disadvantages of protoplast transformation and has proved useful in species previously regarded as untransformable. Electroporation procedures have been developed for many species of lactic acid bacteria[8–14]. For each procedure developed, several parameters had to be tested in order to optimize the transformation efficiency. Some of these parameters are cell preparation conditions, composition of electroporation buffer, voltage and duration of the pulse, regeneration conditions, and type and source of the DNA used. Although electroporation is the method of choice for transformation of lactic

acid bacteria, it cannot be performed by small and less-equipped laboratories because of the expensive equipment required.

In this study, a simple, inexpensive and reliable transformation method for lactic acid bacteria was developed based on agitation of mutanolysin-treated bacterial cells with a vortex mixer in the presence of glass beads, DNA, and polyethylene glycol. Several parameters were tested for their effects on transformation. Transformation of several strains of lactic acid bacteria by this method was also examined in this study.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains of lactic acid bacteria used in transformation experiments were *Lactobacillus casei* ATCC 393, *Lactobacillus fermentus* ATCC 14931, *Lactobacillus reuteri* ATCC 53608, *Leuconostoc dextranicum* ATCC 19255, *Leuconostoc lactis* ATCC 19256, and *Pediococcus acidilactici* ATCC 12697. All of them were grown in MRS medium at 37 °C. Transformants of lactic acid bacteria were cultured at 37 °C in MRS medium containing erythromycin at the final concentration of 3 µg/ml. *Escherichia coli* DH5α harbouring the pGK12 was grown at 37 °C with shaking in LB medium containing erythromycin at a final concentration of 50 µg/ml. Frozen bacterial stock cultures were maintained at −20 °C in appropriate medium plus 20% (v/v) glycerol. Cultures revived from frozen stocks were subcultured twice (2% inoculum) prior to use in experiments.

Plasmid and DNA manipulations

The plasmid used in this study was pGK12. It is a 4.4 kb *E. coli*/*Lactococcus* shuttle vector carrying an erythromycin resistance gene. It was maintained and propagated in *E. coli* DH5α.

Isolation of plasmid from *E. coli* and lactic acid bacteria was done by the alkaline lysis method[15] and the method described by Anderson and McKay[16], respectively. Agarose gel electrophoresis of isolated DNA was performed on 0.8% (w/v) agarose gel in Tris-acetate buffer at 60 V. The DNA was visualized and photographed on an UV transilluminator after staining with ethidium bromide solution (0.5 µg/ml) for 15 min. For Southern hybridization, DNA in the agarose gel was transferred onto Hybond-N^+ nylon membrane (Amersham, UK) using the method described by Sambrook et al[15]. The pGK12 used as a probe was labelled with the DIG High-Prime DNA labelling kit (Roche Diagnostics, USA) following the instructions of the manufacturer.

Electroporation

Electrotransformation of lactic acid bacteria with pGK12 was performed as follows: to obtain electrocompetent cells, bacterial cultures were grown in 50 ml of MRS with 0.5 M sorbitol overnight at 37 °C, and then transferred (2% inoculum) to 800 ml of growth medium (MRS with 0.5 M sorbitol, 3% glycine, and 40 mM DL-threonine). Cultures were incubated at 37 °C to mid log phase. Bacterial cells were harvested by centrifugation at 5000g for 5 min, washed twice in 25 ml of washing solution (0.5 M sorbitol, 10% glycerol), and suspended in 1 ml of electroporation buffer (0.5 M sorbitol, 1 mM K_2HPO_4, 1 mM $MgCl_2$, pH 7.0). The electrocompetent cells were stored in aliquots of 80 µl at −20 °C until use. To introduce plasmid DNA into electrocompetent cells by electroporation, 80 µl suspensions of electrocompetent cells were mixed with 1 µg of pGK12 and the mixture was transferred to an ice-cooled electroporation cuvette (1 mm electrode gap). A single pulse was delivered by Hybaid CelljecT Pro (Hybaid Ltd.) set at 200 Ω resistance and 25 µF capacitance, with a field strength of 1.8 kV/cm. Immediately following the discharge, the suspension was mixed with 1 ml of recovery medium (MRS with 0.5 M sorbitol, 20 mM $MgCl_2$, and 2 mM $CaCl_2$) and left on ice for about 5 min. The cells were allowed to recover for 1 h at 37 °C before spreading a 0.1 ml aliquot of the suspension on selective medium (MRS agar containing 0.5 M sorbitol and erythromycin at the final concentration of 3 µg/ml). Transformants were counted after 5 to 7 days of incubation at 37 °C. Transformation frequency was expressed as erythromycin resistant colonies per 1 µg of pGK12, and values reported represented the mean from 5 separate experiments.

Basic protocol of glass bead transformation

To obtain mutanolysin-treated cells of lactic acid bacteria, a culture of the bacteria was grown at 37 °C in 30 ml of MRS medium to mid log phase. Cells were harvested by centrifugation at 5000g for 5 min, washed in distilled water, and suspended in 5.0 ml of 0.5 M sorbitol in 0.01 M Tris-HCl (pH 7.0). The cell suspension was mixed with mutanolysin (Sigma) (at the final concentration of 50 µg/ml) and then incubated at 37 °C for 30 min. Mutanolysin-treated cells were pelleted at 5000g for 5 min, washed in 10 ml of transformation buffer (0.5 M sorbitol, 0.02 M maleate, 0.02 M $MgCl_2$, pH 6.5), and resuspended in 1 ml of transformation buffer.

Transformation of lactic acid bacteria using glass beads was conducted as follows. Aliquots of 0.5 ml of mutanolysin-treated cell suspension were placed in 15 ml conical disposable polypropylene centrifuge tubes (Corning). To mutanolysin-treated cells, we added 1 µg of plasmid DNA, and immediately after, 500 µl of 30% polyethylene glycol 6,000 (PEG 6000). Then, 0.3 g acid washed glass beads (212–300 µm in diameter, Sigma) sterilized by baking at 250 °C for 2 to 3 h were added. Mutanolysin-treated cells were agitated at the highest speed on a vortex mixer for 15 s, and then diluted by the addition of 10 ml of transformation buffer. After the beads were allowed to settle, the agitated mutanolysin-treated cell suspension was transferred to a new 15 ml conical tube. The cells were pelleted by centrifugation at 5000g for 5 min and suspended in 1 ml of MRS medium supplemented with 0.5 M sorbitol. To allow phenotypic expression of the plasmid encoded erythromycin resistance, the mutanolysin-treated cell culture was incubated 37 °C for 1 h. Transformants were recovered by plating a 0.1 ml aliquot of the mutanolysin-treated cell culture onto MRS agar containing 0.5 M sorbitol and erythromycin at the final concentration of 3 µg/ml and incubated at 37 °C for 5 to 7 days. Transformation frequency was expressed as erythromycin resistant colonies per 1 µg of pGK12, and values reported represented the mean from 5 separate experiments.

Examination of the effects of parameters on transformation

A number of parameters were tested for their effect on transformation. These parameters included the growth phase of the lactic acid bacteria (lag phase, early log phase, mid log phase, late log phase, and stationary phase), the amount of plasmid DNA (ranging from 0.5–5.0 µg), the amount (ranging from 0–0.5 g) and size of glass beads (150–212 µm, 212–300 µm, 425–600 µm, and 710–1180 µm in diameter), the concentration (0, 5, 10, 15, 20, 25, and 30%) and molecular weight (3350, 6000, and 8000) of PEG, the agitation time (between 0 and 30 sec), and the incubation time (between 0 and 60 min) before plating transformants on selective medium. The basic protocol of glass bead transformation was optimized and used to introduce pGK12 into mutanolysin-treated cells of several strains of lactic acid bacteria including *L. casei* ATCC 393, *L. fermentus* ATCC 14931, *L. reuteri* ATCC 53608, *L. dextranicum* ATCC 19255, *L. lactis* ATCC 19256, and *P. acidilactici* ATCC 12697.

Effect of mutanolysin treatment on bacteria survival

Bacteria were grown at 37 °C in 10 ml of MRS medium to mid log phase. Cells were harvested by centrifugation at 5000g for 5 min, washed in distilled water, and suspended in MRS medium with or without 0.5 M sorbitol to obtain the cell concentration of 10^6 CFU/ml. The cell suspension was mixed with mutanolysin (at the final concentration of 50 µg/ml) and then incubated at 37 °C for 30 min. After incubation, the numbers of bacterial cells were determined.

Effect of agitation on survival of bacteria

The optimized protocol of glass bead transformation was performed without using pGK12. Before agitation, the concentrations of mutanolysin-treated cells were adjusted to 10^6 CFU/ml. After agitation, the numbers of bacterial cells were determined.

RESULTS

Electrotransformation with pGK12

Transformation by electroporation was performed with *L. casei* ATCC 393, *L. fermentus* ATCC 14931, *L. reuteri* ATCC 53608, *L. dextranicum* ATCC 19255, *L. lactis* ATCC 19256, and *P. acidilactici* ATCC 12697 to evaluate their ability to be used as recipients for pGK12. Transformation frequencies were obtained (Table 1). The presence of pGK12 in the transformants was confirmed by extraction of the plasmid from 15 representatives obtained from each transformation experiment. All of them were shown to harbour a plasmid DNA whose migration in agarose gels was identical to that of pGK12. The presence of pGK12 in transformants was also confirmed by Southern hybridization analysis using DIG-labelled pGK12 as a probe. It was found that all of the plasmid DNA isolated from the representative transformants co-migrated with pGK12 and hybridized to the probe. Since the electrotransformation of *L. dextranicum* ATCC 19255 with pGK12 gave the highest transformation frequency, it was used as a major recipient for pGK12 in transformation using glass beads.

Transformation of *L. dextranicum* with pGK12 using glass beads

In this experiment, *L. dextranicum* was transformed with pGK12 using the glass bead method. Erythromycin resistant transformants were recovered at the frequency of 323 per µg of pGK12. Plasmid DNA isolated from 15 randomly selected transformants co-migrated in agarose gels with pGK12 and could be hybridized with DIG-labelled pGK12.

Table 1 Numbers of transformants and transformation frequencies obtained from electroporation and glass bead transformation of lactic acid bacteria with pGK12.

Bacteria	Number of transformants ($\times 10^3$)[a]		Transformation frequency ($\times 10^3$)[b]	
	electroporation	glass bead	electroporation	glass bead
L. casei	5.8, 7.4, 3.6, 4.5, 6.3	0.43, 0.4, 0.48, 0.5,0.46	5.52 ± 1.34	0.30 ± 0.02
L. fermentus	6.3, 8.4, 5.3, 7.9, 5.8	0.55, 0.58, 0.46, 0.53, 0.49	6.74 ± 1.20	0.35 ± 0.03
L. reuteri	0.95, 0.87, 0.79, 1.05, 1.12	0.26, 0.38, 0.33, 0.3, 0.35	0.96 ± 0.12	0.21 ± 0.03
L. dextranicum	8.6, 9.4, 7.8, 10.2, 8.1	0.58, 0.61, 0.48, 0.57, 0.5	8.82 ± 0.88	0.37 ± 0.03
L. lactis	3.5, 3.2, 4.4, 3.9, 4.0	0.49, 0.38, 0.45, 0.4, 0.47	3.80 ± 0.41	0.29 ± 0.03
P. acidilactici	4.9, 5.2, 4.2, 4.7, 3.9	0.25, 0.18, 0.28, 0.21, 0.19	4.58 ± 0.47	0.15 ± 0.03

[a] obtained from 5 separate experiments
[b] per µg of pGK12

Table 2 Survival rates of mutanolysin-treated cells in the presence and absence of the osmotic stabilizer (OS, 0.5 M sorbitol) and after agitation (AA).

Bacteria	without OS (%)	with OS (%)	AA (%)
L. casei	0.60 ± 0.10	42.2 ± 0.4	33.1 ± 0.7
L. fermentus	1.60 ± 0.03	57.0 ± 0.3	35.0 ± 0.5
L. reuteri	0.25 ± 0.04	26.1 ± 0.1	27.9 ± 0.7
L. dextranicum	0.23 ± 0.03	63.2 ± 0.3	38.8 ± 0.5
L. lactis	0.42 ± 0.02	31.5 ± 0.2	31.2 ± 0.7
P. acidilactici	0.08 ± 0.02	12.5 ± 0.2	25.9 ± 0.5

mean $\pm$ SD values of 5 replicates

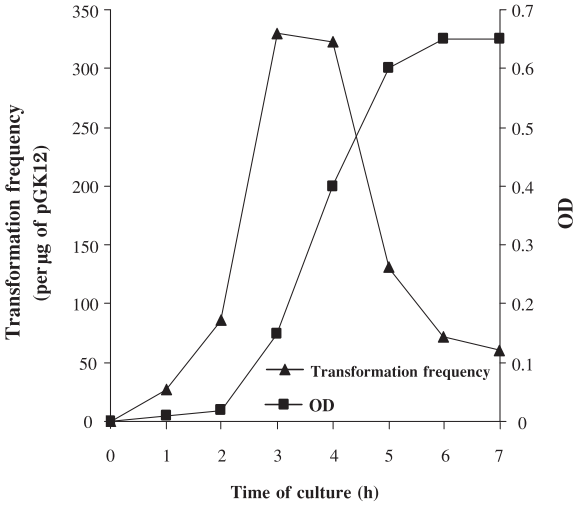

Fig. 1 Effect on transformation frequency of the growth phase of the *L. dextranicum* culture used to prepare mutanolysin-treated cells.

Effect of mutanolysin treatment on survival of bacteria

The survival rates of mutanolysin-treated cells in the presence and absence of the osmotic stabilizer (0.5 M sorbitol) are presented in Table 2. In all tested lactic acid bacteria, survival rates of mutanolysin-treated cells in MRS medium without 0.5 M sorbitol were much lower than those in MRS with 0.5 M sorbitol. These results suggest that mutanolysin can digest cell walls of all tested bacteria.

Optimization of parameters influencing glass bead transformation

The basic protocol of glass bead transformation that successfully introduced pGK12 into *L. dextranicum* mutanolysin-treated cells was modified to determine conditions that would improve transformation frequency. Parameters evaluated included growth phase of bacteria, amount of plasmid DNA, amount and size of glass beads, concentration and molecular weight of PEG, agitation time and incubation time before plating transformants on selective medium.

The transformation frequency was highly dependent on growth phases of bacterial cells to be converted to mutanolysin-treated cells. Glass bead transformation using mutanolysin-treated cells prepared from early log phase and mid log phase cultures gave similar transformation frequencies. However, use of mutanolysin-treated cells from lag phase, late log phase, and stationary phase decreased transformation frequencies (Fig. 1).

The transformation frequencies were somewhat stable at the highest level when 1.5–3.0 µg of DNA was used. Interestingly, increasing of the amount of pGK12 above 3.0 µg resulted in higher numbers of transformants recovered but lower transformation frequencies Fig. 2.

The transformation frequencies using glass beads with diameters of 150–212 µm, 212–300 µm and 425–600 µm were very similar. The highest transformation frequencies were observed when 0.3–0.4 g of any of

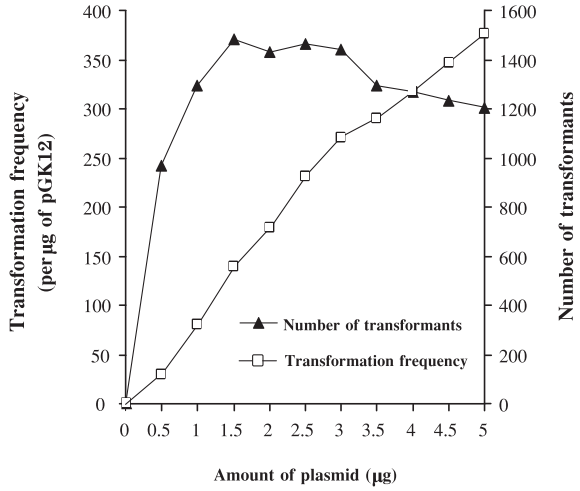

Fig. 2 Effect of amount of plasmid on glass bead transformation of *L. dextranicum* mutanolysin-treated cells.

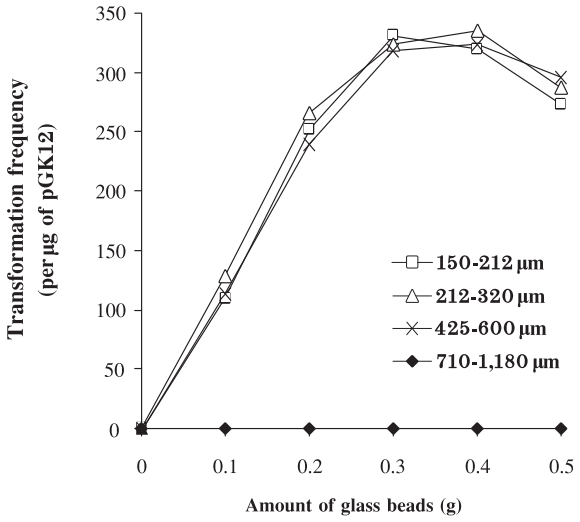

Fig. 3 Effect of amount and size of glass beads on the transformation of *L. dextranicum* mutanolysin-treated cells with pGK12.

these sizes of glass beads was used. No transformant was recovered in transformation using glass bead with diameters of 710–1180 µm (Fig. 3).

Molecular weight of PEG (in the size range tested) was shown not to have any effect on transformation. Transformation frequencies obtained from glass bead transformation of *L. dextranicum* using PEG 3350, PEG 6000, and PEG 8000 were indistinguishable (Fig. 4). However, the transformation frequency was found to be affected by the concentration of PEG. The highest transformation frequency was observed in the transformation using 10% PEG.

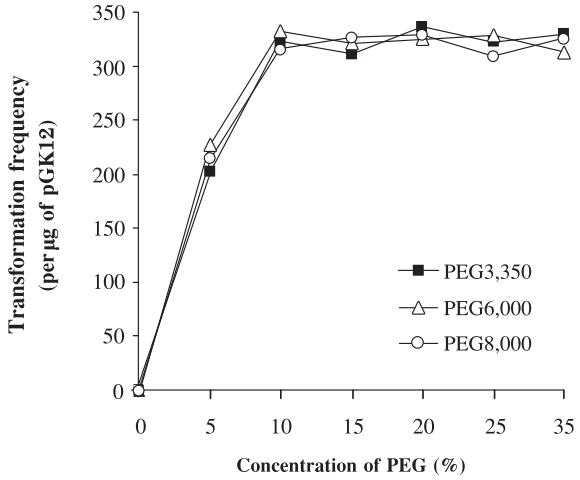

Fig. 4 Effect of concentration and molecular weight of PEG on glass bead transformation of *L. dextranicum* mutanolysin-treated cells with pGK12.

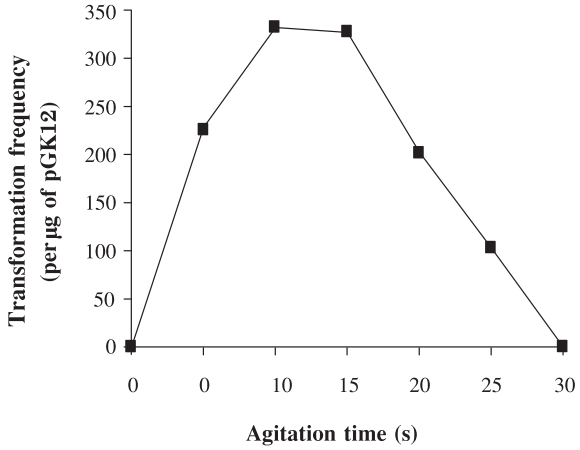

Fig. 5 Effect of agitation time on glass bead transformation of *L. dextranicum* mutanolysin-treated cells with pGK12.

Concentrations of PEG above 10% did not further increase the transformation frequency (Fig. 4).

Effect of agitation time on transformation was also evaluated. Transformation was most efficient between 10 and 15 s of agitation. No transformants were recovered in the glass bead transformation without agitation or with agitation for 30 s (Fig. 5). Mutanolysin-treated cells of *L. dextranicum* that were also mixed with PEG 6000 (15%) and pGK12 (1 µg) without any agitation for 10, 20, 30, 40, 50, or 60 min gave no transformants. The results suggest that the transformation cannot occur naturally and the agitation of mutanolysin-treated cells with glass beads is essential.

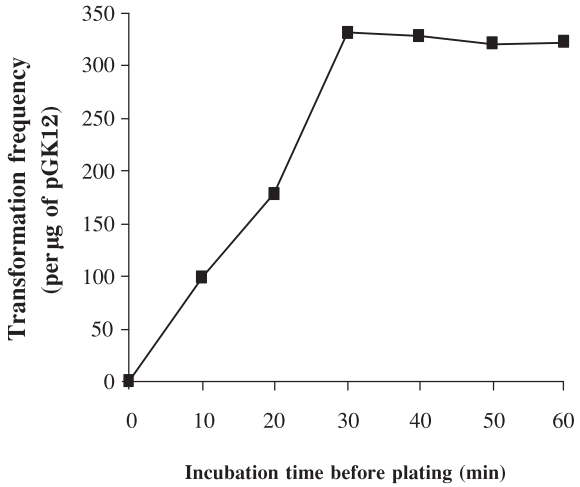

Fig. 6 Effect on transformation frequency of time used to incubate transformants after agitation of *L. dextranicum* mutanolysin-treated cells with pGK12 for 15 s.

The transformation frequency reached a maximum when erythromycin resistance phenotype of pGK12 was allowed to be expressed for at least 30 min before selection on selective plates. Incubation for more than 30 min caused no further increase of transformation frequency (Fig. 6). The incubation of transformants in a nonselective broth seemed to allow the transformants to recover and express the antibiotic resistance, but not to allow them to multiply.

Glass bead transformation of other lactic acid bacteria

The optimized protocol of glass bead transformation was used to introduce pGK12 into mutanolysin-treated cells of several strains of lactic acid bacteria including *L. casei* ATCC 393, *L. fermentus* ATCC 14931, *L. reuteri* ATCC 53608, *L. dextranicum* ATCC 19255, *L. lactis* ATCC 19256, and *P. acidilactici* ATCC 12697. Mutanolysin-treated cells prepared from early log phase culture of each lactic acid bacteria were agitated for 10 s with 0.3 g of glass beads (212–300 µm in diameter) in the presence of 1.5 µg of pGK12 and 10% PEG 6000. Transformants were allowed to recover in MRS supplemented with 0.5 M sorbitol for 30 min at 37 °C before plating on selective agar medium. It was found that glass bead transformation could be used to introduce pGK12 into all strains of lactic acid bacteria used in this study (Table 1). Transformation frequencies and numbers of transformants obtained from these experiments show that this transformation method has acceptable efficiency and good reproducibility.

Effect of agitation on survival of bacteria

The survival rates of mutanolysin-treated bacterial cells were determined after agitation with 0.3 g of glass beads (212–300 µm in diameter) in the presence of 10% PEG 6000 for 10 s (Table 2). The agitation was shown to kill more than 60% of mutanolysin-treated cells of all lactic acid bacteria tested.

DISCUSSION

In the development of the glass bead transformation, selection of the plasmid and lactic acid bacteria used is very important. The selected plasmid DNA must contain at least one selectable marker and be able to replicate in selected lactic acid bacteria. In this study, pGK12 plasmid was chosen to transform several selected strains of lactic acid bacteria including *L. casei* ATCC 393, *L. fermentus* ATCC 14931, *L. reuteri* ATCC 53608, *L. dextranicum* ATCC 19255, *L. lactis* ATCC 19256, and *P. acidilactici* ATCC 12697. All of them have been previously transformed with pGK12 by electroporation[14]. The pGK12 is a small (4.4 kb) plasmid containing two antibiotic resistance determinants: chloramphenicol resistance determinant (*cat* gene) and erythromycin resistance determinant (*erm* gene), both of which are readily selectable and/or scoreable markers. Plasmid pGK12 replicates in a variety of Gram-positive bacteria as well as in *E. coli*[17].

The ability of pGK12 to transform all lactic acid bacteria used in this study was confirmed by electroporation. Based on the high transformation frequencies obtained from electroporation, *L. dextranicum* was selected as the main recipient of pGK12 to develop and optimize the glass bead transformation. By using the basic protocol of glass bead transformation, pGK12 was successfully introduced into mutanolysin-treated cells of *L. dextranicum* with a frequency of 323 transformants per µg of plasmid. To our knowledge, this is the first report showing a successful transformation of lactic acid bacteria with a plasmid by glass bead method. The number of transformants recovered from 5 separate transformations of *L. dextranicum* with pGK12 by glass bead method and by electroporation (Table 1) indicated that both transformation methods provided high reproducibility, even though electroporation gave higher transformation frequency.

As in several reports concerning protoplast transformation of Gram positive bacteria[5,6], glass bead transformation was influenced by the growth phase at which they were taken before the mutanolysin treatment. The highest transformation frequencies were obtained from glass bead transformation of *L. dextranicum* with pGK12 when mutanolysin-treated cells

prepared from early log phase and mid log phase cells were used. They may be more tolerant to physical stress by agitation with glass beads and/or have better recovery ability than those prepared from other growth phases.

The amount of plasmid DNA used in glass bead transformation is one of the major factors determining the transformation frequency. The highest transformation frequencies occurred when 1.5–3.0 µg of plasmid DNA was used. If the amount of plasmid DNA was increased over 3.0 µg, the transformation frequencies decreased. It is not known if this is due to an inappropriate proportion of the amount of mutanolysin-treated cells to the amount of DNA used in the transformation. A similar result was found in the protoplast transformation of *Streptococcus faecalis* OG1RF with pGB354[6]; an increase of plasmid concentration beyond 0.5 µg per ml of transformation mixture caused a reduction of transformation frequency. However, in the protoplast transformation of *Streptococcus lactis* LM0230 with pGB301, transformation frequency was found to be proportional to DNA concentration[7].

Transformation of *L. dextranicum* with pGK12 using glass beads with sizes between 150–600 µm in diameter resulted in similar transformation frequencies with the highest frequencies occurring when 0.3–0.4 g of glass beads were used. However, no transformant was recovered in the transformation using glass beads with size of 710-1180 µm in diameter which provided spaces between beads big enough to allow most mutanolysin-treated cells not to directly contact the beads. The glass beads size did not affect cell survival (data not shown).

PEG treatment has been proved to be essential for protoplast transformation of Gram positive bacteria. The mechanism by which PEG induces transformation is still unclear, but it may cause the cell membrane to become more permeable to DNA, or it may cause a conformational change in the DNA molecule which allows penetration into the mutanolysin-treated cell. In this study, concentration of PEG but not its molecular weight seemed to affect the transformation. The optimal concentration of PEG for glass bead transformation was found to be greater than or equal to 10% which is lower than that for protoplast transformation of Gram positive bacteria. The optimal concentrations of PEG for protoplast transformation of *Streptomyces* spp., *Streptococcus lactis*, *Bacillus subtilis*, and *Streptococcus faecalis* OG1X were reported to be 20%[18], 22.5%[7], 30%[18], and 40%[6], respectively. Agitation of mutanolysin-treated cells with glass beads may be the reason for the reduction of concentration of PEG used in the transformation.

In summary, we would like to stress that this is the first report of the transformation of lactic acid bacteria using glass beads. Because of its convenience, cost saving, high reproducibility, and acceptable efficiency, it may be an alternative method for the transformation of lactic acid bacteria with plasmid DNA. Further work in our laboratory aims to improve this glass bead transformation method and to use it with other Gram positive bacteria.

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