Efficient transformation of *Lactobacillus sake* by electroporation

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A procedure to transform intact *Lactobacillus sake* cells by electroporation was developed through a systematic examination of the effect of changes in various parameters on the transformation efficiency of *Lact. sake* strain 64F. The most critical factors were found to be the electrical parameters, the composition of washing and electroporation/storage solutions, and the presence of MgCl₂ in the expression medium. Under optimal conditions transformation efficiencies up to 10⁷ transformants (µg supercoiled DNA)⁻¹ were obtained. The optimized procedure was successfully applied to other *Lact. sake* strains and consistently yielded from 10⁴ to 10⁷ transformants (µg supercoiled DNA)⁻¹.

Keywords: *Lactobacillus sake*, electroporation, genetic transformation, vectors

INTRODUCTION

Lactobacillus sake is an industrially important lactic acid bacterium used as a starter culture in the processing of dry fermented sausages and other fermented meat products (Hammes *et al.*, 1990). It contributes to several steps in meat processing, influencing colour, flavour and texture, and enhancing the hygienic stability of the final product (Lücke & Hechelmann, 1987; Berdagué *et al.*, 1993; Hammes & Knauf, 1994; Verplaetse, 1994). Because of these properties, this bacterium is receiving increasing attention. To apply molecular genetic techniques to the analysis of this micro-organism, an efficient transformation system is required. Several groups have attempted to electrotransform *Lact. sake* (Gaier *et al.*, 1990; Aukrust & Blom, 1992; Aymerich *et al.*, 1993), reporting frequencies ranging from 10² to 10⁴ transformants (µg DNA)⁻¹.

This paper describes the optimization of an electroporation procedure for *Lact. sake*, which routinely yields between 10⁴ and 10⁷ transformants (µg supercoiled DNA)⁻¹, depending on strains and plasmids used. The procedure was developed with *Lact. sake* strain 64F and plasmid pGK12 by systematically investigating the effect of various parameters on transformation efficiency. Factors analysed were: growth conditions, washing and electroporation/storage solutions, expression medium, DNA topology and concentration, and electrical parameters. Conditions were established for the successful transfer of various plasmids into plasmid-cured strains of *Lact. sake* as well as wild-type strains of meat origin.

METHODS

Bacterial strains, growth conditions and plasmids. The *Lact. sake* strains used in this study were isolated from meat or meat products. *Lact. sake* 160x1, G3, GV1, 23, 55, 64, 112, 179 and 207 were from our laboratory collection; *Lact. sake* LTH 939 and LTH 945 were kindly provided by Dr W. P. Hammes, Institute of Food Technology, Stuttgart, Germany. *Lact. sake* 23K and 160x1K are plasmid-free strains derived from strains 23 and 160x1, respectively, and 64F and G3AF are partially cured strains derived from strains 64 and G3, respectively (see Results). *Lact. sake* strains were subcultured on MRS medium (de Man *et al.*, 1960) and incubated at 30 °C. *Escherichia coli* and *Lactococcus lactis* subsp. *lactis* strains carrying plasmid vectors were grown on LB medium (Sambrook *et al.*, 1989) at 37 °C and on M17 medium containing 0.5% (w/v) glucose (Terzaghi & Sandine, 1975) at 30 °C, respectively. When present, antibiotics were added at the following concentrations (µg ml⁻¹): erythromycin (5) and chloramphenicol (10) for *Lact. sake* and *L. lactis*; ampicillin (100) for *E. coli*. Stock cultures were maintained at -20 °C in the presence of glycerol. Fresh cultures were prepared from frozen stocks through two transfers in MRS, M17 or LB broth.

Plasmids pNZ12, pIL253 and pGK12 were isolated from *L. lactis* subsp. *lactis* IL1403 and pULP8 from *E. coli* TG1, using the alkaline lysis procedure described by Birnboim & Doly (1979). Plasmid DNA from transformed *Lact. sake* strains was isolated by the method of Anderson & McKay (1983). Purification of plasmid DNA by CsCl/ethidium bromide density gradient centrifugation was as described by Sambrook *et al.* (1989).

DNA techniques. All DNA manipulations and gel electrophoresis procedures were carried out as recommended by the suppliers or by standard methods (Sambrook *et al.*, 1989).

DNA/DNA hybridization was performed according to Southern (1975) using [α - 32 P]dCTP (Amersham) nick-translated plasmid DNA probes.

Plasmid curing. *Lact. sake* strains were grown in 10 ml MRS broth in the presence of novobiocin ($1\text{--}128\ \mu\text{g ml}^{-1}$) at $37\ ^\circ\text{C}$ for 96 h. Cultures containing the highest novobiocin concentration (usually $32\ \mu\text{g ml}^{-1}$) which still allowed visibly detectable growth were plated on MRS agar at appropriate dilutions. The presence or absence of plasmids was detected by DNA extraction and hybridization with radioactively labelled plasmid probes.

Electroporation. Electroporation was carried out with a Gene Pulser and a Pulse Controller apparatus (Bio-Rad).

Selection of transformants. Transformants were selected by plating on MRS agar with the appropriate antibiotic and incubating for 48 h at $30\ ^\circ\text{C}$. Control suspensions without DNA were also spread in duplicate onto MRS agar plates and selective media to determine surviving c.f.u. and the spontaneous reversion frequency. Transformation efficiency and transformation frequency were defined as transformants $(\mu\text{g DNA})^{-1}$ and transformants per surviving cells.

RESULTS

Plasmid curing

Curing experiments were carried out with wild-type *Lact. sake* strains 23, 64, 160x1 and G3. These strains had different plasmid contents as revealed by electrophoresis on agarose gel (not shown). The number of different plasmid species present in strains 23, 64, 160x1 and G3 was 1, 2, 2 and 4, respectively. Their size ranged from 1 to 50 kb. Novobiocin treatment, combined with growth at sublethal temperature ($37\ ^\circ\text{C}$), generated a plasmid-free strain 23 (named 23K), and eliminated one plasmid in strains 160x1 and 64 (these partially cured strains were named 160x1A and 64F, respectively), and two plasmids in strain G3 (named G3AF). Attempts were made to eliminate the remaining plasmids using other curing agents (incompatibility, electroporation, pH). The single plasmid of strain 160x1A was eliminated after transformation with plasmid pNZ12. This plasmid shared homology with pNZ12, as deduced by Southern hybridization (not shown), which supports the hypothesis that the two were incompatible. pNZ12 was lost by subculturing the cells for 200 generations in MRS at $30\ ^\circ\text{C}$ without selection (the plasmidless strain 160x1 was named

160x1K). In the case of strains 64F and G3AF, no cured derivatives were obtained by these treatments.

Optimization of electrotransformation of *Lact. sake*

Currently available cloning vectors for lactic acid bacteria are based either on broad-host-range theta-replicating plasmids such as pAM β 1 (Simon & Chopin, 1988) or on small, rolling-circle replicating lactococcal or lactobacilli plasmids. As the starting point from which to develop a procedure for transformation of *Lact. sake*, we have taken the two *L. lactis* vectors pGK12 (Kok *et al.*, 1984) and pNZ12 (de Vos, 1986) based on the rolling-circle-type replicons of pWV01/pSH71, because their broad host-range has allowed the construction of a large number of cloning vectors in all genera of lactic acid bacteria; the theta-type plasmid pIL253 (Simon & Chopin, 1988), a high-copy-number derivative of pAM β 1, because it may accommodate inserts of large size; the *Lact. plantarum*/*E. coli* shuttle vector pULP8, a derivative of the *Lact. plantarum* natural plasmid pLP1, because it also has the advantage of being able to promote high-copy-number plasmid replication in *E. coli* (Bringel & Hubert, 1990). The *Lact. sake* strains 23K, 160x1K and 64F were initially electroporated with the four plasmids as described by Langella *et al.* (1993) for *L. lactis*, except that M17 broth was replaced by MRS broth. Transformants were recovered at efficiencies ranging from 10^2 to 10^4 $(\mu\text{g DNA})^{-1}$, depending on the strain and plasmid used (data not shown). Moreover, a considerable variation in transformation efficiency was obtained with different competent cell preparations of the same strain. Therefore, this protocol was systematically modified to allow identification of parameters influencing transformation efficiencies. For this purpose, we used *Lact. sake* strain 64F and plasmid pGK12, which usually gave the highest efficiency [approximately 10^4 transformants $(\mu\text{g supercoiled DNA})^{-1}$]. The electrical parameters in the experiments described below were $12.5\ \text{kV cm}^{-1}$ (peak voltage), $25\ \mu\text{F}$ capacitance and $200\ \Omega$ resistance, unless otherwise indicated.

(a) Effect of the composition of washing and electroporation/storage solutions. The composition of the washing and electroporation/storage solutions tested is indicated in Table 1. *Lact. sake* 64F cells washed with various solutions were mixed with plasmid pGK12 and

Table 1. Effects of the composition of washing and electroporation/storage solutions on the transformation efficiency of *Lact. sake* 64F cells by pGK12 DNA

Washing solution	Electroporation/storage solution	Transformants $(\mu\text{g DNA})^{-1}$
10 mM MgCl_2	0.5 M Sucrose/10% glycerol	8.8×10^5
	30% PEG 3000/10% glycerol	$< 10^2$
	10% Glycerol	7.0×10^4
	0.5 M Sucrose/10% glycerol/3.5 mM MgCl_2	8.5×10^4
0.5 M Sucrose/10% glycerol	0.2 M Sucrose/10% glycerol	9.0×10^5
	0.5 M Sucrose/10% glycerol	1.4×10^4
	30% PEG 3000/10% glycerol	$< 10^2$

Fig. 1. Effect of MgCl_2 in the washing solution on the transformation efficiency of *Lact. sake* 64F cells. Cells were prepared and electroporated with pGK12 plasmid DNA under standard conditions (see text), except that they were washed with solutions containing 0–50 mM MgCl_2 . The electroporation settings were 2.5 kV, 25 μF and 200 Ω (time constant: 4.3 or 4.4 ms).

electroporated under standard conditions. Similar results were obtained with plasmids pIL253 and pNZ12 (data not shown). PEG has been reported to increase the efficiency of protoplast transformation (Morelli *et al.*, 1987) and electroporation of *Lact. casei* (Chassy & Flickinger, 1987; Natori *et al.*, 1990), *Lact. plantarum* (Bringel & Hubert, 1990) and *Lact. sake* (Aukrust & Blom, 1992), and is commonly used for *L. lactis* electroporation (Langella & Chopin, 1989). However, 30% (w/v) PEG 3000 as electroporation/storage solution completely inhibited electrotransformation of *Lact. sake* 64F cells. Furthermore, the addition of Mg^{2+} (3.5 mM MgCl_2), a commonly used divalent cation in electroporation buffers, is not recommended since it lowered the efficiency about 10-fold. Interestingly, washing of the cells in 10 mM MgCl_2 prior to electroporation increased the efficiency of transformation by one to two orders of magnitude. The highest efficiency was obtained by washing the cells in 10 mM MgCl_2 , followed by resuspension in a solution containing 0.5 M sucrose (0.2 M sucrose was equally good however) plus 10% (v/v) glycerol (Table 1).

The positive effect of Mg^{2+} concentration in the washing solution on the number of transformants was further investigated. Fig. 1 shows that the addition of Mg^{2+} ions to the washing solution significantly increased the number of transformants (about 10-fold) with a maximum value at 10 mM MgCl_2 . Two washing steps gave a higher number of transformants but three washes gave no further improvement (data not shown). Therefore, cells were routinely washed twice in 10 mM MgCl_2 , stored and electroporated in 0.5 M sucrose plus 10% glycerol.

(b) Effect of the composition of the growth medium. The effect of cell wall weakeners, such as glycine, lysine and threonine, was investigated. *Lact. sake* 64F cells were

grown to an OD_{600} of approximately 0.4–0.6 in MRS broth supplemented with 0.5–3.5% (w/v) glycine, 40 mM threonine, or 40 mM lysine, and in non-supplemented MRS broth as a control. Cells were harvested, washed with the electroporation/storage solution described above and electroporated under standard conditions. No significant change in either cell viability (about 1.9×10^{10} cells $\text{ml}^{-1} \pm 0.5 \times 10^{10}$) or transformation efficiency [1.5×10^6 – 4.5×10^6 transformants (μg pGK12 DNA) $^{-1}$] was observed. Interestingly, the addition of sucrose in the growth medium (0.25 M), a commonly used osmostabilizer in electroporation media, had no effect on the electroporation frequency of *Lact. sake* (data not shown). The cells were therefore routinely grown in non-supplemented MRS broth.

(c) Effect of the growth stage. To investigate the effect of growth stage on *Lact. sake* 64F transformation efficiency, the cells were harvested at different OD_{600} values (0.2, 0.4, 0.6 and 0.8, corresponding to early-, mid- and late-exponential phase, respectively) prior to electroporation. The cell suspensions were then adjusted to a final concentration of approximately 1.7×10^{10} cells ml^{-1} in the electroporation/storage solution. Aliquots were mixed with pGK12 plasmid DNA and the mixtures were immediately used for electroporation. From 60 to 70% of the cells survived the electric pulse. Irrespective of cell density, the transformation efficiency ranged from 1.0×10^6 to 4.0×10^6 transformants (μg pGK12 DNA) $^{-1}$, indicating that the growth phase did not influence the efficiency significantly. The cells to be electroporated were subsequently grown to mid-exponential phase ($\text{OD}_{600} = 0.4$ – 0.6).

(d) DNA concentration and topology. To examine the effect of DNA concentration on the number of transformants, various amounts of CsCl-purified pGK12 plasmid DNA (10 ng to 8 μg) were added to 50 μl portions of competent *Lact. sake* 64F cells. DNA/cell mixtures were electroporated under standard conditions (12.5 kV cm^{-1} , 25 μF and 200 Ω). As shown in Fig. 2, the number of transformants was proportional to the DNA concentration over the range used in this experiment [0.2–160 μg DNA (ml cells) $^{-1}$]. Under these conditions, the mean transformation efficiency was 2.5×10^6 transformants (μg pGK12 DNA) $^{-1}$ and the highest number of transformed cells represented about 1% of the viable cells. Similar results were obtained with plasmid pNZ12 over the range 0.8–128 μg DNA (ml cells) $^{-1}$ (data not shown). Interestingly, a linear dose–response relationship was also observed using the theta-replicating plasmid pIL253, albeit with a much narrower DNA concentration range of 0.2–8 μg DNA (ml cells) $^{-1}$. The reasons for this are unclear and were not investigated further. In subsequent trials, we used a DNA concentration of 1 μg ml^{-1} .

DNA purity appears to be an important factor since pGK12 plasmid DNA from minipreps obtained through the alkaline-lysis method gave few transformants [about 10^3 (μg DNA) $^{-1}$]. Ligation mixtures did transform, at about 5×10^5 transformants (μg ligated DNA) $^{-1}$, compared to 1×10^7 transformants (μg supercoiled pGK12 DNA) $^{-1}$. The following conditions were used.

Fig. 2. Effect of DNA concentration on transformation efficiency of *Lact. sake* 64F cells. Electrocompetent cells were prepared and electroporated as described in the legend to Fig. 5, except that electrical parameters were 2.5 kV, 25 μ F and 200 Ω (time constant: 4.3 or 4.4 ms). The cells were transformed with 0.2–160 μ g CsCl-purified pGK12 plasmid DNA (●) ml⁻¹ or with 0.2–100 μ g CsCl-purified pL253 plasmid DNA (○) ml⁻¹.

pGK12 DNA, digested with restriction enzymes to give either cohesive or blunt-ended fragments, was ligated by T4 DNA ligase, extracted once with phenol/chloroform, and ethanol-precipitated. Competent 64F cells (50 μ l) were transformed with 50 ng ligated DNA. The electrical parameters were 9 kV cm⁻¹, 25 μ F and 600 Ω .

(e) Electrical parameters. To determine the effect of varying electrical settings on transformation efficiencies, cells were electroporated under various voltage and pulse control resistance combinations at a capacitance of 25 μ F. Competent cells for electroporation were prepared according to the optimized conditions defined above and in section (f) below. Results for *Lact. sake* strain 64F electroporated with plasmid pGK12 DNA are represented as a three-dimensional surface plot in Fig. 3. The plot has an optimum from high voltage/low resistance (short pulses) to low voltage/high resistance (long pulses). Along the optimum shown in the plot, the combinations 1.8 kV/600 Ω and 1.5 kV/1000 Ω gave the highest efficiencies, 1.2×10^7 and 1.0×10^7 transformants (μ g DNA)⁻¹, respectively. In contrast, the number of transformants decreased drastically at low voltage/low resistance, or high voltage/high resistance, which shows that the electrical parameter setting is an important determinant in the recovery of transformants. Therefore, in the final protocol, trials were done with a pulse at 600 Ω , 25 μ F and 1.8 kV, which gave a time constant between 11.3 and 13.8 ms.

(f) Expression medium and expression time. Before plating, the electroporated *Lact. sake* 64F cells were incubated in the expression medium (MRS broth) at 30 °C for various times (2–20 h). A minimum of 2 h incubation was required (not shown), to obtain the optimal ratio of transformants to survivors. Further incubation (6 h) gave

Fig. 3. Response surface plot showing transformation efficiency of *Lact. sake* 64F cells as a function of voltage and pulse control. Electrocompetent cells were prepared and electroporated with pGK12 DNA under the optimal conditions described in the legend to Fig. 5. Transformation efficiency is indicated by ●. The plot data were obtained by the RSREG and G3GRID procedures of SAS system, version 6 (SAS Institute Inc.).

Fig. 4. Effect of MgCl₂ concentration in the expression medium on transformation efficiency of *Lact. sake* 64F cells. Cells were prepared and electroporated with pGK12 DNA as described in the legend to Fig. 2. Expression was achieved after incubation for 2 h in MRS broth supplemented with MgCl₂.

a twofold higher number of transformants but also a twofold increase in survivors, due to cell division. A period of 20 h incubation led to an increase in cell death. The addition of MgCl₂ in the expression medium considerably improved the transformation results (Fig. 4).

Fig. 5. Outline of the optimized protocol for *Lact. sake* electroporation. See text for details. TE buffer: 10 mM Tris/HCl, pH 8.0, 1 mM EDTA. *MRS broth used was supplemented with 10 g glucose l⁻¹.

Expression was achieved in MRS medium supplemented with MgCl₂ (concentrations ranging from 1 to 100 mM), or unsupplemented (MgCl₂ concentration in MRS broth is 0.4 mM) as control. The number of transformants increased drastically (up to 600-fold) with increasing concentrations of MgCl₂, reaching a plateau of 10⁶ (µg pGK12 DNA)⁻¹ for 60 mM MgCl₂. The number of viable cells was not affected by the MgCl₂ concentration (not shown). Other divalent cations were tested: Mn²⁺ (MnSO₄) was as efficient as MgCl₂ up to a concentration of 5 mM, but higher concentrations led to the formation of a precipitate in MRS broth. Ca²⁺ (CaCl₂) up to 2 mM had no effect alone and reduced the efficiency when added in combination with MgCl₂. Therefore, an expression medium supplemented with 80 mM MgCl₂ was routinely used.

Optimized procedure for *Lact. sake* electroporation

Taking into account the results of the experiments described above, the details of the optimized procedure are as outlined in Fig. 5.

(a) Reproducibility. The optimized procedure is readily reproducible: a two-fold variation in transformation efficiency was observed when different aliquots of the same batch of cells were transformed with the same plasmid, and a fivefold variation with transformation of aliquots of different batches.

(b) Transformation by various plasmids. Various plasmids were successfully transferred to *Lact. sake* 64F cells using the optimized procedure (Table 2). High efficiencies [$> 2 \times 10^6$ transformants (µg supercoiled DNA)⁻¹] were obtained with *L. lactis* vectors pGK12 and pNZ12, related to the rolling-circle-type replicons of pWV01/pSH71, and with the theta-type plasmid pIL253, a high-copy-number derivative of pAMβ1. The presence of plasmids in the transformants was confirmed by DNA extraction from randomly chosen clones and electrophoresis (not shown). In contrast, no transformants were recovered with plasmid pULP8, suggesting that this replicon cannot establish in *Lact. sake* strain 64F. The reasons for this were not studied further.

(c) Transformation of other *Lact. sake* strains. Using the conditions established with strain 64F, plasmid-mediated transformation of various other *Lact. sake* strains (plasmid-cured or wild-type) was evaluated. As seen in Table 2, transformation efficiencies ranging from 10⁴ to 10⁷ (µg DNA)⁻¹ were obtained in most experiments, except for one strain (LTH 945) which was not transformable. All the other strains were transformed, either by the four plasmids tested or at least by some of them. Each strain was highly transformed by at least one of the plasmids. Interestingly, comparable efficiencies were obtained with the wild-type strains and their plasmidless derivatives, suggesting that the endogenous plasmids did not interfere with plasmid establishment in the host cell. Some variations in transformation efficiency were observed between strains and plasmids, which may indicate that the different replicons cannot be used effectively in all *Lact. sake* strains. However, these results demonstrate that the transformation procedure developed in this study is suitable for a variety of *Lact. sake* strains and plasmid replicons.

DISCUSSION

In this report, we present the development of an efficient transformation protocol for *Lact. sake*. Up to 10⁷ transformants (µg DNA)⁻¹ can be obtained under optimal conditions (plasmids pNZ12 or pGK12 and strain 64F). These transformation efficiencies are comparable to the highest values reported for other lactic acid bacteria (Holo & Nes, 1989; Watanabe *et al.*, 1994; Wells *et al.*, 1993). Also, they are by far the highest reported for *Lact. sake* (Gaier *et al.*, 1990; Aukrust & Blom, 1992; Aymerich *et al.*, 1993). For instance, cells of *Lact. sake* LTH 939, which yielded only 1.0 × 10² transformants (µg pNZ12 DNA)⁻¹ (Gaier *et al.*, 1990), were electrotransformed with an efficiency of 3.5 × 10⁵ transformants (µg pNZ12 DNA)⁻¹ by using the protocol developed in this study.

Among the factors tested to optimize the transformation of *Lact. sake* cells by electroporation, we found that the

Table 2. Transformation efficiencies of various strains of *Lact. sake* with several plasmids under optimal conditions

Strain	Transformants (μg plasmid DNA) ⁻¹			
	pGK12	pNZ12	pULP8	pIL253
64/64F	1.3×10^7	1.1×10^7	$< 10^2$	2.7×10^6
23/23K	8.2×10^5	8.0×10^6	2.0×10^6	2.3×10^3
160x1/160x1K	1.1×10^4	1.4×10^4	5.4×10^5	1.1×10^4
G3/G3AF	$< 10^2$	1.7×10^6	$< 10^2$	9.3×10^4
55	1.1×10^5	5.4×10^5	1.6×10^5	$< 10^2$
112	9.5×10^4	1.2×10^4	6.3×10^4	3.0×10^3
179	$< 10^2$	1.6×10^4	$< 10^2$	$< 10^2$
207	8.4×10^3	1.4×10^4	$< 10^2$	$< 10^2$
GV1	3.1×10^5	8.9×10^5	$< 10^2$	$< 10^2$
LTH939	1.0×10^5	3.5×10^5	1.8×10^5	4.6×10^4
LTH 945	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$

cell wall may not be a barrier to the entry of DNA in *Lact. sake* 64F since there was no increase in transformation efficiency when cells were grown in a medium containing cell wall weakening agents. In support of this hypothesis, no osmotic stabilizer is needed in the protocol developed here. This was also evident from the high number of surviving cells after electroporation (60–70%). This contrasts with several reports on lactic acid bacteria (for a review, see Gasson & Fitzgerald, 1994), indicating that the presence of strong cell wall weakeners in the growth medium yielded significant improvement in the transformation efficiency.

As observed by Aukrust & Blom (1992), the use of MgCl_2 (10 mM) during the washing of the cells enhanced transformation efficiencies (about 14-fold). This effect has been related to the reduction by MgCl_2 in the amounts of extracellular polysaccharides which seem to have a negative effect on transformation. In agreement with this hypothesis, we observed that *Lact. sake* strain 160x1, producing large amounts of slime, had systematically lower transformation efficiencies than strains 23K and 64F. These differences may be due to incomplete removal of slime by MgCl_2 .

The presence of MgCl_2 (80 mM) in the incubation medium before plating was also critical for recovering a high number of transformants of *Lact. sake* (approx. 600-fold increase), while Aymerich *et al.* (1993) found MgCl_2 decreased the transformation efficiency. The positive effect of MgCl_2 is not due to enhanced post-pulse survival, as proposed by Morelli *et al.* (1987) and Cosby *et al.* (1988) for the regeneration of protoplasts, since the viable cell count was not modified. It may be that MgCl_2 allows a better maintenance of the plasmids in the recipient cell.

Our results also show that the voltage settings and pulse control resistance have a major impact on the process. The three-dimensional plot (Fig. 3) illustrates how the resistance should be adjusted at different voltages to obtain the most efficient electroporation. Thus, at the highest voltage, only short pulses should be used since

increase in the time constant results in extensive cell killing. In contrast, at lower voltage, longer pulses are necessary to get higher numbers of transformants. The response pattern is very similar to those reported by Aukrust & Blom (1992) with various *Lact. plantarum* strains. Optimal parameters obtained in this study are also in agreement with those used for electroporation of *Lact. casei* (Watanabe *et al.*, 1994). The resemblance may suggest that bacterial membranes react in a similar way during electrical pulses.

The total number of transformants recovered increases linearly with DNA concentration over a large range, especially for the test rolling-circle-type replicons, which may be convenient for direct cloning. At the highest DNA concentration used, about 1% of the cells were transformed, indicating a rather high level of electrocompetence.

Most of the *Lact. sake* strains tested were transformed efficiently using the procedure developed here, showing the protocol is well adapted to this bacterial species. Some variations in the transformation efficiencies were observed between strains and plasmids. The failure of some strains (wild-type) to be transformed may be related to resident plasmids that interact with the transforming plasmids. In agreement with these results, we observed that the cryptic plasmid from strain 160x1A was eliminated after the entry of pNZ12 by electroporation in *Lact. sake* 160x1. This plasmid is related to the pNZ12 replicon. In addition, *Lact. sake* LTH945 and 179, harbouring the highest number of plasmids (3), yielded the lowest efficiency, whereas the two cured-strains 160x1K and 23K were transformed efficiently. However, no change in the number of transformants was obtained between these two plasmidless strains and their wild-type ancestors. Therefore, poor transformation results might be due to inability of the test replicons to establish in the host cells.

In conclusion, the transformation protocol described here differs in several ways from those described in previous reports on lactic acid bacteria. It constitutes a reproducible

and efficient method for introducing plasmid DNA into *Lact. sake* and should therefore facilitate gene manipulations in this species.

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