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New method for electroporation of *Lactobacillus* species grown in high salt

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ABSTRACT

We here describe a new method for electroporation of *Lactobacillus* species, obligately homofermentative and facultatively heterofermentative, based on the cell-wall weakening resulting from growth in high-salt media. For *L. casei*, optimum transformation efficiency of up to 10^5 transformants per microgram of plasmid DNA was achieved following growth in the presence of 0.9 M NaCl. Plasmids of different sizes and replication origins were also similarly transformed. These competent cells could be used either directly or stored frozen, up to 1 month, for future use, with similar efficiency. This protocol was assayed with different *Lactobacillus* species: *L. delbrueckii* subsp. *lactis*, *L. paracasei*, *L. plantarum* and *L. acidophilus*, and it was found that they were transformed with similar efficiency.

1. Introduction

Bacteria of the genus *Lactobacillus* have considerable industrial importance. They are well known for their widespread application in various food and agricultural fermentation processes (Hammes and Hertel, 2006). They are currently being considered as new vehicles for antigen delivery tools for oral vaccination (Adachi et al., 2010; Hanniffy et al., 2004; Pouwels et al., 1998). Therefore, there is an increasing interest in the manipulation and improvement of *Lactobacillus* strains, using modern genetic engineering techniques (Kondo and Johansen, 2002).

Limited transformability is in part consequence of their thick cell wall, which is highly resistant to mechanical disruption. In particular, a high resistance to cell-wall hydrolases like lysozyme or mutanolysin is observed in several lactobacilli species (Piuri et al., 2005).

A variety of techniques have been assayed in order to improve the electro-transformation of lactobacilli (Aukrust et al., 1995). Treatments with lysozyme, glycine or penicillin, which allow reducing the intrinsic wall resistance, have been shown to improve the electro-transformation efficiency of particular strains (Aymerich et al., 1993; Chassy and Flickinger, 1987; Mason et al., 2005; Thompson and Collins, 1996; Wei et al., 1995).

We have previously observed in *L. casei* BL23 that growth in high salt increases its sensitivity to lysis, probably as a consequence of the decreased peptidoglycan cross-linking (Piuri et al., 2005). In fact, osmotic stress induced by a high salt concentration has been reported to increase cell autolytic activity and survival to lyophilization of *L. delbrueckii* subsp. *lactis* (Koch et al., 2007).

In the present work, we investigated whether the growth in high salt can render the wall of *Lactobacillus casei* strain fragile enough to increase its electro-transformability. We describe an inexpensive and simple protocol involving NaCl addition to the growth medium as a cell-wall-weakening agent. In addition, we assayed several *Lactobacillus* species, obligately homofermentative and facultatively heterofermentative, in order to validate this new transformation protocol. We selected species containing or not endogenous plasmids to investigate their influence on the electro-transformability. We also assayed strains endowed of surface layer protein (S-layer) to verify its effect in the transformation procedure.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study were laboratory stocks obtained from CERELA-CONICET (Tucumán, Argentina), INLAIN-UNL (Santa Fe, Argentina) and IATA-CSIC (Valencia, Spain) and kept frozen with 15% (v/v) glycerol at −20 °C.

Plasmid-free strains are: *Lactobacillus casei* BL23 (cured of endogenous plasmid pLZ15), *Lactobacillus delbrueckii* subsp. *lactis* LKT, *Lactobacillus acidophilus* ATCC 4356 and *Lactococcus lactis* MG1363. Plasmid harboring strains are: *Lactobacillus plantarum* ATCC 14917, *L. paracasei* ATCC27092 and ATCC27139.

Strains were grown at 37 °C in MRS medium (Biokar, France) (Hammes and Hertel, 2006) either containing or not NaCl at the concentration of 0.9 M. When required, due to a temperature-sensitive replicating plasmid or osmosensitive strains the incubation temperature was 30 °C and the NaCl concentration was 0.7 M, respectively.

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Escherichia coli strains KW1 [*metB strA purB*(*aad-uid-man*) *hsr hsm*$^+$ *gusA*$^-$] and JM109 [*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(rk−, mk+), *supE*44, *relA*1, Δ(*lac-proAB*)/F′ [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15] were grown in LB, incubated at 37 °C and used to amplify the plasmids.

2.2. Plasmids and DNA isolation

The plasmids used and their characteristics are shown in Table 1. Plasmid DNA was isolated by the procedure of Birnboim and Doly (1979); this procedure was modified for lactic acid bacteria as described by Chassy and Flickinger (1987) and used for plasmid screening of the transformants obtained with the different *Lactobacillus* strains. Restriction endonucleases (Fermentas) were used to identify the restriction patterns of the plasmids, according to manufacturer. DNA fragments were resolved on 0.8% agarose gel electrophoresis.

2.3. Preparation of electrocompetent cells

Precultures of *Lactobacillus* were obtained in MRS medium incubated without aeration at 37 °C for 18 h. Competent cultures were obtained after inoculating 1 ml of the preculture to 100 ml MRS medium containing NaCl at the concentration of 0.9 M, and then further incubated. For osmosensitive strains the NaCl concentration was 0.7 M. When OD_{600nm} reached 2–2.5, growth was stopped by cooling cultures on ice for 10 min. The cells were harvested by cool centrifugation and washed three times with ice-cold ultra pure water. Cells were resuspended in 1 ml of water (100-fold concentration of cell suspension), resulting in a total count of about 10^{12} CFU/ml and fractions of 50 μl were either used directly for electroporation or stored at −70 °C for up to 1 month for further use. LAB protocol in Table 1 was according to Chassy and Flickinger (1987).

Table 1
Electroporation efficiencies obtained in *Lactobacillus casei* BL23 using several plasmids.

Plasmid/host (origin)[a]	Relevant characteristics (size, marker, replication mode, and host range)	Electroporation protocol[b]		
		Transformation efficiency CFU/μg		
		M1	M2	LAB[c]
pIL253/ *L. casei*	4.8 kb Em^R Theta replication	$2.8 \pm 0.1\ 10^4$	$1.2 \pm 0.1\ 10^5$	$5.5 \pm 1.1\ 10^4$
pG$^+$host9/ *L. lactis*[d]	3.75 kb Em^R Rolling circle		$6.0 \pm 0.1\ 10^6$	
pNZ273/*E. coli* KW1	4.66 kb Cm^R Rolling circle	$2.8 \pm 1.3\ 10^3$	$5.7 \pm 0.4\ 10^5$	$1.7 \pm 0.2\ 10^4$
pGK13/*E. coli* JM109	4.96 kb Cm^R, Em^R Rolling circle Broad host range	$1.3 \pm 0.1\ 10^5$	$5.9 \pm 0.1\ 10^5$	
pRV610/*E. coli* JM109	5.17 kb Em^R, Ap^R Theta replication		$5.2 \pm 1.2\ 10^5$	
pRV613/*E. coli* JM109	8.69 kb Em^R Ap^R Theta replication		$1.43 \pm 0.1\ 10^6$	
pRV620/*E. coli* JM109	5.6 kb Cm^R Ap^R Theta replication		$1.8 \pm 0.1\ 10^5$	

[a] pNZ273is a pSH71 derivative (Platteeuw et al., 1994; de Vos and Simons, 1988); pGK13 (Kok et al., 1984); pRV610, pRV613 and pRV620 are derivatives of pRV500, a natural plasmid of *L. sakei* 332 (Alpert et al., 2003; Crutz-Le Coq and Zagorec, 2008); pIL253 is a pAMβ1 derivative (Simon and Chopin, 1988; Bruand et al., 1993); pG$^+$Host9 is a temperature-sensitive pWV01 replicating plasmid (Maguin et al., 1992). These plasmids were kindly provided by M. Zagorec and A. Gruss from INRA Jouy en Josas (France); strains KW1 and JM109 were (rk−, mk+) as relevant phenotypes.

[b] Transformants/μg is the mean of three independent transformation assays and standard deviation.

[c] The electroporation protocol was according to Chassy and Flickinger (1987).

[d] Transformants were counted after incubation at 30 °C.

2.4. Electroporation protocols

Electroporation was carried out using a Gene Pulser electroporator (Bio-Rad, Hemel Hempstead, UK). 50 μl of competent cells were mixed with 5 μl plasmid DNA (50 to 100 ng/experiment) in 0.2-cm electrode gap cuvettes (Bio-Rad, UK) and subjected to the following electrical parameters: applied voltage (KV), parallel resistance (R in Ω), capacitance (C in μF), field strength (V/cm), Time constant (τ in ms). Electrical settings for protocol M1 were 2 KV, 400 Ω, 25 μF, 10,000 V/cm, 10 ms and for protocol M2, 2.5 KV, 200 Ω, 25 μF, 12,500 V/cm, 5 ms.

Following electroporation, cells were mixed with 0.95 ml MRS medium and incubated at 37 °C for 3 h. About 100 μl of a 1/5 dilution was plated on MRS agar medium with the resistance encoded antibiotics (either chloramphenicol (Cm 5 μg/ml) or erythromycin (Erm 5 μg/ml)). After 48–72 h of anaerobic incubation, transformants were counted. Strictly anaerobic growth conditions are required to avoid a second stressor as oxidative stress would be. In all cases, transformants were analyzed for plasmid DNA content and restriction analysis by agarose electrophoresis. At least three independent transformation assays were performed in each experiment. The transformation efficiencies were expressed as the number of transformants (CFU) per microgram of plasmid DNA, and standard deviation determined.

3. Results and discussion

L. casei BL23 (cured of plasmid pLZ15) was chosen to investigate the different parameters concerning the development of a new electroporation procedure. Two different electroporation protocols were tested with two combinations of electrical settings (voltage and resistance) at a capacitance of 25 μF.

Many factors can influence the transformation efficiency; some are linked to the plasmid origin, size and replication mechanisms, whereas others are linked to the characteristics of the strains used. Table 1 shows the results of the electroporation efficiencies with the different plasmids and parameters evaluated. Protocol M2 (see Section 2.4) yielded the best performance, giving consistent efficiencies of up to 10^5 transformants/μg plasmid DNA for *L. casei*, using the higher electroporation field strength of 12.5 kV cm^{-1}.

In a first approach, we compared the electroporation efficiencies with six different plasmids of different sizes, selection markers, copy number per cell and replication mechanism. We did not observe (Table 1) any significant variation, and the efficiencies remained reproducibly high regardless of the origin, marker, and size of the plasmids in the *L. casei* strain used. We also analyzed the plasmid source since restriction–modification systems can inhibit transformation with foreign DNA (Alegre et al., 2004). For this purpose, plasmid pNZ273, prepared from a *L. casei* transformant, was used to electroporate the plasmid-free strain. Again, 6×10^5 transformants/μg of plasmid DNA were obtained, the same efficiency as that obtained when the plasmid was isolated from *Escherichia coli*.

The size of the plasmid was also evaluated. The same replication origin for pRV500 derivatives of different sizes resulted in similar efficiencies (compare pRV610 or pRV620 with pRV613 in Table 1).

In all cases, plasmid screening of the transformants was performed by restriction analysis of the extracted plasmids, to verify that no size modification occurred due to rearrangements generated by the endogenous restriction–modification systems. Fig. 1A shows samples of the results of plasmid pNZ273 extracted from *L. casei* transformants were size was preserved according to the electrophoretic mobility observed.

A key factor of this procedure was the requirement of high cell density, since, when less than 10^{10} CFU/ml were used, low levels of transformation were obtained (the efficiency decreased to $2 \pm 0.1\ 10^3$ for pRV610 and to $4.7 \pm 0.1\ 10^3$ for pNZ273).

To determine the efficiency of our procedure, we compared our results with those of other published electroporation protocols.

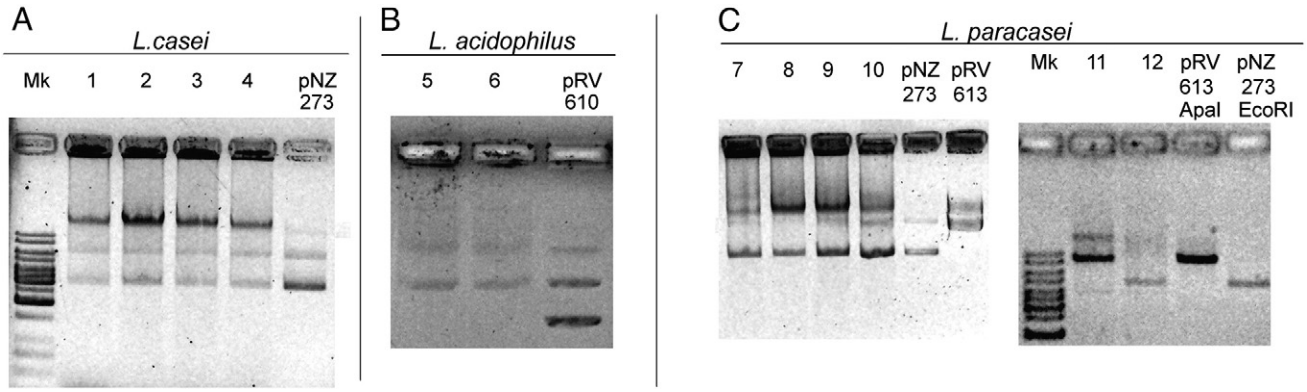

Fig. 1. Analysis of transformants by plasmid extractions. Transformants in *L. casei* (panel A), *L. acidophilus* (panel B), and *L. paracasei* (panel C) were selected, and their plasmid content analyzed by agarose gel electrophoresis, as indicated in Section 2.2. The numbers indicate individual isolates from each species. Controls vectors are indicated. In panel C coexistence of the two plasmids was corroborated by restriction analysis. Lanes 11 and 12 are the same clones that were digested with ApaI (lane 11) and EcoRI (lane 12), respectively.

For *L. casei*, the reported efficiencies varied from 4.5×10^3 (Chassy and Flickinger, 1987) to 2.0×10^4 (Mason et al., 2005) transformants/μg, whereas, when we have reproduced this same protocol (Table 1, LAB protocol) as a direct comparison, it yielded efficiencies 10-fold lower that those obtained with our procedure.

Since one important feature is the reproducibility and linear relationship between plasmid input and number of transformants, we assayed several plasmid concentrations and electrotransformed fresh competent cells of *L. casei*; in such experiments, we observed a linear relationship between number of transformants and the plasmid DNA concentration (Fig. 2), which resulted in a constant efficiency of up to 10^5 transformants/μg plasmid.

In order to verify whether frozen-stored competent cells kept their transformability, experiments were performed to confirm the possibility to keep competent cells frozen for further use. Similar values to the ones obtained with fresh cells were observed (Table 2 and Fig. 2). Although freezing decreased viability by one order, it did not result in loss or reduced electro-transformability. Since we did not add any cryo-protectant to freeze the competent cultures, we suggest that the compatible solutes accumulated during the growth in high salt (glycine–betaine and peptides; Piuri et al., 2003), may act as cryoprotectants, for storage at −70 °C. In view of these findings an advantage of this procedure is potential freeze storage, that it is a good option to use competent cells within the following days or weeks, and up to 1 month.

It is known that transformability can be strain- and species-dependent (Serror et al., 2002; Alegre et al., 2004). In order to look whether this procedure can be applied to other *Lactobacillus* species, *L. delbrueckii* subsp. *lactis*, *L. plantarum*, and two *L. paracasei* strains were assayed. As shown in Table 3, the procedure was also efficient with these species. For thermophilic bacteria like *L. delbrueckii* subsp. *lactis* an additional thermal treatment has been described to weaken the cell wall more effectively (Serror et al., 2002). Watanabe et al. (1994) reported efficiencies of up to 1×10^7 per μg DNA for *L. paracasei* ATCC 27092 (formerly *L. casei*) that are comparable with those obtained with this new procedure (Table 3).

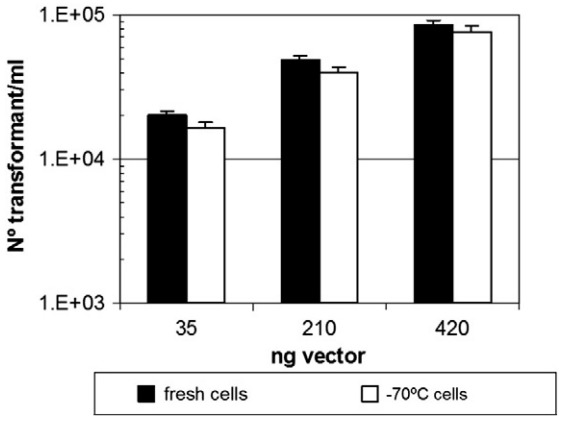

Fig. 2. Effect of increasing plasmid DNA on transformation efficiency. Competent cells, fresh (filled bars) or frozen-stored (empty bars), from *L. casei* were electrotransformed with DNA from pNZ273 plasmid as reported in Table 3. CFU obtained in chloramphenicol plates were counted and reported for each plasmid DNA input.

The presence of plasmids in *Lactobacillus* strains which may reduce the transformation efficiency (due to incompatibility or rearrangements) was studied by means of two approaches: the use of strains containing native plasmids and the use of a *Lactobacillus* strain transformed with a plasmid as a recipient cell for a second transformation. In the first case, the use of plasmids with a selection marker is a clear advantage. The yields obtained with *L. plantarum* harboring a plasmid were higher (Table 3) than those reported previously (Thompson and Collins, 1996; Alegre et al., 2004; Mason et al., 2005), thus indicating that the presence of a native or endogenous plasmid does not interfere negatively. In the second approach, the use of two different plasmid replication systems (rolling circle or theta) and positive selection markers for each was also advantageous. *L. paracasei* transformed with pNZ273, was made electrocompetent and electroporated with pRV613, with an efficiency of $4.2\pm0.2\,10^6$ similar to that reported for one plasmid only (Table 1). To verify the presence of the two individual plasmids in the double transformant, the plasmids extracted were digested with ApaI to linearized pRV613 and EcoRI to linearized pNZ273. As shown in Fig. 1C, the coexistence of the two plasmids was corroborated by restriction analysis. Neither the endogenous nor the introduced plasmids reduced the transformation efficiency. We did not investigate the incompatibilities which are known to take place (Posno et al., 1991), since this aspect was out of the scope of the present work.

Table 2
Effect on transformability of freeze storage.

	Fresh Cell	Stored −70 ° C
	Cell counts (CFU/ml)	
Total viable	$1.8\,10^{12}$	$8.0\,10^{11}$
Post pulse Viable	$2.0\,10^{9}$	$4.0\,10^{9}$
% survival	0.1%	0.5%
ng plasmid pNZ273	Transformation efficiency (CFU/μg)	
35	$5.7\,10^{5}$	$4.7\,10^{5}$
210	$2.3\,10^{5}$	$1.9\,10^{5}$
420	$2.0\,10^{5}$	$1.8\,10^{5}$

Lactobacillus casei BL23 was used for evaluation of freeze storage.
Fresh and frozen competent cells were electrotransformed with DNA from pNZ273 plasmid. From each DNA input, the CFU obtained in chloramphenicol plates were counted and efficiencies (CFU/μg) calculated.

Table 3
Electroporation efficiencies of other *Lactobacillus* species.

	Transformation efficiency (CFU/μg)[a]		
Plasmid	pNZ273	pRV610	pGK13
Strain			
L. delbrueckii subsp. *lactis* LKT		$1.4 \pm 0.1\,10^5$	$2.0 \pm 0.1\,10^5$
L. plantarum ATCC 14917	$9.2 \pm 0.3\,10^5$	$2.6\,10^5$	
Lact. paracasei ATCC 27092	$3.3 \pm 0.2\,10^6$		
Lact. paracasei ATCC 27139	$4.8 \pm 0.3\,10^6$		
L. acidophilus ATCC 4356		$1.4 \pm 0.2\,10^5$	$2.5 \pm 0.3\,10^4$

[a] Transformants/μg is the mean of three independent transformation assays with fresh cells and protocol M2. *L. delbrueckii* subsp. *lactis*, *L. acidophilus* and *L. plantarum* were grown at 0.9 M NaCl, while *L. paracasei* strains were grown at 0.7 M NaCl.

Growth in high salt (0.7–0.9 M NaCl) is a general stressor applicable to a larger number of *Lactobacillus* species. However, the NaCl concentrations allowing enough growth need to be optimized for each new strain. While *L. delbrueckii* subsp. *lactis* and *L. plantarum* were able to grow in high salt concentration (0.9 M NaCl), *L. paracasei* strains were more osmosensitive and were grown at 0.7 M NaCl, a concentration that efficiently weakened the cell wall, as shown by the transformation efficiency in Table 3.

Several species of the genus *Lactobacillus* possess an S-layer which may increase their resistance to high-salt weakening. We are currently investigating *L. acidophilus* bearing S-layer (Prado-Acosta et al., 2008; 2010) and its relationship to high-salt adaptation. When electro-transformation was performed in this strain according to the procedure here described, we obtained comparable efficiency of transformants (Table 3 and Fig. 1B) to those previously reported for other *L. acidophilus* strains (Walker et al., 1996; Kim et al., 2005). In fact, in our laboratory, with the conditions and protocol described in Kim et al. (2005), we were unable to reproduce their efficiencies.

In summary, we described a new procedure for efficiently electroporate a wide range of lactobacilli species and strains, which consists essentially in weakening their cell wall by growth in hypertonic NaCl-containing medium. Comparable and even higher efficiencies of transformants were obtained when compared to the other cell-wall-weakening agents-based protocols (lysozyme, glycine or penicillin) previously described. Competent frozen cells conserved their transformation capacity. No need of addition of cryoprotectants for freeze storage was required. Inexpensive NaCl addition is an advantage of this method as compared with the other cell-wall-weakening agents (lysozyme, glycine or penicillin) previously described (Chassy and Flickinger, 1987; Aymerich et al., 1993; Thompson and Collins, 1996; Alegre et al., 2004; Wei et al., 1995; Mason et al., 2005).This method only requires small adjustments for determining NaCl concentrations allowing enough growth and incubation time needed must be optimized for each new strain.

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