Transfer of plasmid DNA to *Brevibacterium lactofermentum* by electrotransformation

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The *Escherichia coli*–*Brevibacterium lactofermentum* shuttle vector pBLA was introduced into intact cells of *B. lactofermentum* by electrotransformation. Several parameters of this procedure such as voltage and cell concentration were analysed. Optimal conditions gave an efficiency of 10^6 transformants per μg of DNA. Two recalcitrant strains could be electrotransformed when an ampicillin pretreatment step was used. Electrotransformation experiments using DNAase or different structural forms of plasmid DNA showed that the electrotransformation process is quite different from natural transformation involving competence development. Restriction–modification-proficient *B. lactofermentum* could be efficiently electrotransformed with pBLA DNA isolated from *E. coli*. This restriction–modification system therefore seems to be overcome by electrotransformation. Thus electrotransformation may efficiently replace the protoplast bacterial transformation method.

Introduction

Molecular genetics has undergone a tremendous development as a result of the discovery of novel systems for the exchange of genetic material. The first system to be discovered was DNA-induced natural bacterial transformation in *Streptococcus pneumoniae* (Avery *et al.*, 1944). The number of naturally transformable strains being small, several other techniques have been devised to introduce DNA into bacteria. The discovery of penetration of DNA into *Escherichia coli* cells by Mandel & Higa (1970) opened the field of genetic engineering. However, this procedure is inefficient for the majority of bacteria. Protoplast transformation is much more generally applicable to most genera (Bibb *et al.*, 1978; Chang & Cohen, 1979; Katsumata *et al.*, 1984), but it is difficult to establish the best conditions for transformation, and the method is often not reproducible. Moreover, these methods have been hampered by inconsistency, the time taken for the procedure and the low efficiency of transformation. DNA uptake by electrotransformation seems to be a much more general system (see Chassy *et*

al., 1988, for a review), although some strains are recalcitrant to electrotransformation (Chassy *et al.*, 1988; Wirth *et al.*, 1989). As genetic engineering requires the transformation of a variety of strains, it is important to develop a procedure to overcome these difficulties. We report here the plasmid electrotransformation of an amino acid producer, *Brevibacterium lactofermentum*, which we have previously transfected and transformed by the protoplast method (Yeh *et al.*, 1985, 1986), and we describe a protocol that allows electrotransformation of strains that were previously untransformable.

Methods

Bacterial strains and growth conditions. Electrotransformation experiments were performed with three strains of *B. lactofermentum*. Strains 15 and 10 were isolated as restriction–modification mutants of strain 180 (Bonnassie *et al.*, 1990), which is a rifampicin-resistant derivative of *B. lactofermentum* ATCC 21086. They were grown in Luria–Bertani (LB) medium (Maniatis *et al.*, 1982) at 34 °C, with shaking. For the preparation of agar plates, Bacto-agar (Difco) was added to a final concentration of 1.5% (w/v). Media for selection and subsequent growth of transformants contained 20 μg kanamycin sulphate ml^{-1}

(Sigma). *E. coli* AB1157 (F⁻ *thr-1 ara-14 leuB6* Δ (*gpt-proA*)62 *lacY1 tsx-33 supE44 galK2* λ^- *rac hisG4*(Oc) *rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*), derived from *E. coli* K12, was used for plasmid DNA preparation in order to test for restriction in *B. lactofermentum*.

Plasmid isolation. Large-scale preparation of plasmid DNA from *B. lactofermentum* was done by the method of Yeh *et al.* (1986) and from *E. coli* AB1157 according to Ish-Horowitz & Burke (1981). Plasmids were purified by CsCl/ethidium bromide density-gradient centrifugation and stored in water. DNA concentrations were determined by measuring absorbance at 260 nm.

Purification of plasmid DNA by electroelution. All restriction enzymes were obtained from BRL and were used as recommended by the supplier. Plasmid DNA fragments were analysed by 0.8% agarose gel electrophoresis (Maniatis *et al.*, 1982) and purified by electroelution with the IBI Unidirectional and Analytical Electroeluter system. λ DNA cut by *Hind*III, which was purchased from Boehringer Mannheim, was used as a molecular mass marker and as a DNA concentration reference.

Electrotransformation procedures. For electrotransformation with the Gene Pulser apparatus from Bio-Rad, cells were grown in LB medium to an OD₅₄₀ of 0.3–0.5 and harvested by centrifugation. Cells were then washed twice in phosphate/sucrose electroporation buffer (PSB: 7 mM-potassium phosphate pH 7.5, 0.5 M-sucrose, 1 mM-MgCl₂) and concentrated in 0.8 ml PSB (2.5×10^8 c.f.u. ml⁻¹). Unless otherwise indicated, transformation was carried out with 500 ng plasmid DNA per assay, which gave a DNA concentration of 625 ng ml⁻¹. With the Pulse Controller apparatus, 40 μ l of cells (about 7×10^8 c.f.u. ml⁻¹) was mixed with 37.5 ng of plasmid DNA (937.5 ng ml⁻¹). Cells were kept on ice for 10 min before and after the electric pulses.

Electrotransformation with the gene pulser apparatus from Jouan Laboratories was performed using cells washed three times in Tris/sucrose electroporation buffer (TSB: 10 mM-Tris/HCl pH 7.5, 270 mM-sucrose, 0.5 mM-MgCl₂). About 10^8 cells were mixed with 75 ng of pBLA into a final volume of 18 μ l and immediately transferred into the sterile electrode chamber for the pulse. Cells were at once diluted in LB medium for expression before selection.

Results

Construction of the *B. lactofermentum*–*E. coli* shuttle vector pBLA

The plasmid used for electrotransformation was the 10.1 kb *B. lactofermentum*–*E. coli* shuttle vector pBLA. It was obtained by cloning the 5.6 kb *Hind*III fragment of pAT21.1 (obtained from P. Courvalin: Trieu-Cuot & Courvalin, 1983), which carries an *Enterococcus faecalis* gene encoding kanamycin resistance and the ampicillin resistance gene from transposon Tn3, into the *Hind*III site of pBL1 (a 4.4 kb plasmid isolated from a *B. lactofermentum* strain, which was obtained from A. Deschamp) (Fig. 1). As observed for *Corynebacterium glutamicum* (Ozaki *et al.*, 1984), the ampicillin resistance gene does not allow the phenotypic selection of *B. lactofermentum* transformants.

Effect of voltage on survival and transformation frequency

For this study we used *B. lactofermentum* strain 15, which is a restriction- and modification-deficient derivative of *B. lactofermentum* 180. To determine suitable conditions for electrotransformation of *B. lactofermentum* 15, we examined the effect of the initial electric field strength on transformation frequency and survival.

For field strengths of 3.25 to 5 kV cm⁻¹, the transformation frequency (transformants per viable cell) increased up to 1.4×10^{-4} (Fig. 2a). Within the range 5 to 6.25 kV cm⁻¹ (the latter is the maximum that can be obtained by the Bio-Rad Gene Pulser unit) the transformation frequency remained essentially constant. No cell death was observed (data not shown). The DNA concentration used in these experiments was 625 ng ml⁻¹. As the number of transformants is proportional to DNA concentration up to more than 1 μ g ml⁻¹ (Bonnassie *et al.*, 1989), the transformation efficiency can be evaluated as 4.8×10^4 per μ g DNA. Field strengths greater than 6.25 kV cm⁻¹, obtained with the Pulse Controller unit, produced a significant decrease in transformation frequency and survival fell to 44% at 12.5 kV cm⁻¹ (Fig. 2b).

The experiments described above were done using exponential decay voltage pulses (Bio-Rad system). We also used the Jouan apparatus to provide square wave pulses, which allows control of physical parameters such as pulse duration. We used two field strengths (4.7 and 5.3 kV cm⁻¹) and pulse durations of 3, 5, 7 and 10 ms. The highest transformation frequency was 2.8×10^{-5} transformants per viable cell and occurred at a field strength of 4.7 kV cm⁻¹ and a pulse duration of 10 ms. The frequencies obtained in the other treatments differed at most by a factor of three (data not shown).

Effect of cell concentration

In the preceding experiments a cell concentration of 2×10^8 c.f.u. ml⁻¹ was arbitrarily chosen. However, the yield of transformants might be increased with an increase in the number of cells present when the DNA concentration is held constant, as reported for electrotransformation of *E. coli* (Dower *et al.*, 1988). Using the same amount of DNA (500 ng), we tested this expectation (Table 1). High cell concentrations increased the number of transformants. Although the transformation frequency was nearly constant, due to the linear response between transformants and cell concentration, the use of a higher cell concentration (3×10^9 c.f.u. ml⁻¹) increased the transformation efficiency to 8×10^5 transformants per μ g of DNA. These results are in agreement with experiments reported by Wolf *et al.* (1989) and Bonamy *et al.* (1990) on *Corynebacterium glutamicum*.

Fig. 1. Construction of the pBLA *B. lactofermentum*-*E. coli* shuttle vector. This plasmid was constructed from pBL1 (*B. lactofermentum* plasmid) and pAT21.1 (*E. coli* plasmid). Ap and Km signify the genes for ampicillin and kanamycin resistance respectively.

Fig. 2. Effect of field strength on transformation frequency. Cell suspension of *B. lactofermentum* 15 (2×10^8 c.f.u. ml⁻¹) was mixed with pBLA DNA (625 ng ml⁻¹). (a) Dependence of transformation frequency (transformants per viable cell, □) on field strength in the range 3.125 to 6.25 kV cm⁻¹ (25 µF capacitor) was determined using the gene pulser apparatus (Bio-Rad) with electrodes of 0.4 cm gap (0.8 ml cell suspension). (b) High voltage densities were achieved using the Pulse Controller unit, electroporation cuvettes with a 0.2 cm interelectrode distance (40 µl cell suspension) and field strengths between 6.25 and 12.5 kV cm⁻¹ (25 µF capacitor). The transformation frequency (□) and the survival (●) are shown.

Table 1. Effect of cell concentration on transformation

pBLA (500 ng) was added to 0.8 ml of a suspension of *B. lactofermentum* 15 (1.0×10^8 to 3×10^9 c.f.u. ml⁻¹). The cells were pulsed at 6.25 kV cm⁻¹ (25 µF capacitor). Transformants were selected on a medium containing kanamycin (25 µg ml⁻¹). The frequency (transformants per viable cell) and the transformation efficiency (transformants per µg of DNA) were determined.

C.f.u. ml ⁻¹ (x)	Transformants ml ⁻¹ (y)	Transformation frequency (y/x)	Transformation efficiency
1.0×10^8	1.5×10^4	1.5×10^{-4}	2.4×10^4
4.0×10^8	1.0×10^5	2.5×10^{-4}	1.6×10^5
3.0×10^9	5.0×10^5	1.7×10^{-4}	8.0×10^5

Electrotransformation of cells pre-treated with ampicillin

The experimental conditions established to transform *B. lactofermentum* 15 gave poor results for the parental strain 180 and strain 10. For these refractory strains, the intact cell wall may hinder DNA uptake. Partial disorganization of the cell wall, by pre-treatment with sublethal concentrations of a β -lactam antibiotic, could render refractory strains electrotransformable. Indeed, it has been reported that protoplast transformation also required such pretreatment associated with a lysozyme treatment (Katsumata *et al.*, 1984; Yeh *et al.*, 1985; Yoshihama *et al.*, 1985).

Exponentially growing cells were treated with different concentrations of ampicillin for 90 min (one doubling time), then electrotransformed as described in Methods. The two refractory strains could be transformed only when ampicillin was added to the cell culture (Fig. 3a, b). This pretreatment also slightly improved the transformability of strain 15 (Fig. 3c). However, the level of transformation obtained for the two refractory strains was never as high as that for strain 15. The optimal concentration of ampicillin was 0.5 to 1.5 µg ml⁻¹. With ampicillin treatment and without electric pulse no transformants could be detected.

Evidence for plasmid DNA penetration during the electric pulse

To investigate whether cells remain permeable to DNA after the electric pulse, DNAase (8 µg ml⁻¹) was added to the samples (2×10^8 c.f.u. ml⁻¹, 875 ng DNA ml⁻¹) at different stages of electrotransformation. DNAase completely inhibited transformation when added before the electric pulse, but had no inhibitory effect (1.4×10^4 transformants ml⁻¹ obtained) when added immediately after the pulse. This shows that DNA uptake is very fast and suggests that DNA penetration is concomitant with the electric pulse.

Fig. 3. Electrotransformation of cells pretreated with ampicillin. DNA (500 ng) was added to 0.8 ml of cell suspension of *B. lactofermentum* strain 180 (a), 10 (b) or 15 (c). The samples were pulsed at 6.25 kV cm⁻¹ (25 µF capacitor) and the numbers of transformants (□) and viable cells (●) were determined.

Electrotransformation of cells by different structural forms of plasmid DNA

pBLA was linearized with restriction enzyme *Pst*I, which cuts within the β -lactamase gene (Fig. 1). Linear fragments and open-circle forms were purified by

Table 2. Influence of electric field on the restriction-modification system

The transformation efficiencies (transformants per μg of DNA) of *B. lactofermentum* strains 15 (r^-m^-) and 180 (r^+m^+) with pBLA (500 ng) isolated from either *E. coli* or *B. lactofermentum* were determined. For electrotransformation, the cell suspensions of *B. lactofermentum* strains 180 (2×10^7 c.f.u. ml^{-1}) and 15 (3×10^7 c.f.u. ml^{-1}) were pretreated with ampicillin ($1 \mu\text{g ml}^{-1}$) and concentrated 10-fold before the pulse (6.25 kV cm^{-1} , $25 \mu\text{F}$ capacitor).

Recipient	Transformation efficiency pBLA from:	
	<i>B. lactofermentum</i>	<i>E. coli</i>
<i>B. lactofermentum</i> 180 (r^+m^+)	1.0×10^4	1.5×10^3
<i>B. lactofermentum</i> 15 (r^-m^-)	1.6×10^6	3.0×10^5

electroelution and used for electrotransformation. Denatured DNA samples were also prepared. The open-circle forms transformed efficiently (2.2×10^5 transformants per μg DNA). About the same efficiency was obtained with the native pBLA DNA. However, the use of either linear or denatured forms of pBLA decreased the transformation efficiency (respectively 2.0×10^3 and 3.4×10^4 transformants per μg DNA). Presumably either the uptake of linear or denatured DNA is reduced or these forms are degraded readily after entry.

Influence of electric field on the restriction-modification system

We studied the electrotransformation of strains 180 (r^+m^+) and 15 (r^-m^-) by pBLA isolated from either *E. coli* or *B. lactofermentum*. Table 2 shows that the transformation efficiency of strain 180 using heterologous plasmid DNA was about 10-fold lower than that obtained using homologous plasmid DNA. A similar difference was found when strain 15 was transformed using homologous and heterologous plasmid DNA. In contrast, with the PEG-mediated-transformation procedure, heterologous transformation was reduced 900-fold as compared with homologous transformation for *B. lactofermentum* strain 180 and only 10-fold for strain 15 (Bonnassie *et al.*, 1990). Thus restriction by *B. lactofermentum* 180, which has been lost in the r^-m^- strain 15, could not be detected by electrotransformation.

Discussion

We have shown that plasmid DNA can be efficiently introduced into intact cells of *B. lactofermentum* by

electrotransformation. This method is more rapid and gives a more reproducible transformation frequency (transformants per viable cell) than conventional procedures, which require the conversion of cells to protoplasts followed by a regeneration step.

The data obtained with *B. lactofermentum* 15 (r^-m^-) indicate that an initial electric field strength of 6.25 kV cm^{-1} , with a capacitance of $25 \mu\text{F}$, gives an efficiency of 4.8×10^4 transformants per μg DNA for a cell concentration of 2×10^8 c.f.u. ml^{-1} . This efficiency can be increased with cell concentration up to 8×10^5 transformants per μg DNA. Other parameters such as growth conditions or electroporation buffer composition could be important in electrotransformation, but they were not analysed in this work. MacNeil (1987) observed a relationship between the transformation frequency and the lysis of protoplasts. In contrast, as previously described by Miller *et al.* (1988), we found that lethality does not appear necessary to obtain efficient transformation in intact cells. This is supported by the fact that the greatest yield of transformants occurred when the survival rate was high. Our results indicate that electrotransformation is a process quite different from transformation via the development of natural competence; it does not require special conditions of growth and DNA uptake is extremely fast, as if DNA penetration were concomitant with the pulse. This suggests that the transport of DNA across the cell envelope occurs during the electric pulse.

Pre-treatment with ampicillin allowed electrotransformation of two recalcitrant *B. lactofermentum* strains, for which no transformants were obtained without pre-treatment. Ampicillin, a member of the β -lactam family, partially disorganizes the cell wall. Presumably the cell wall in these strains hindered DNA uptake during the electric pulse. At the concentrations used here, ampicillin does not lyse the cells, but seems to increase transformability. It is not known whether this is due to an increase in the fraction of transformable cells in the culture or to an increased probability of DNA uptake per viable cell. This ampicillin pre-treatment is a suitable and brief step for transforming some recalcitrant strains. However, the optimal conditions of ampicillin treatment will probably have to be carefully determined for each strain.

Restriction of heterologous DNA was not detected by electrotransformation. One explanation could be that DNA uptake mainly involves single-stranded DNA, which escapes restriction, but this is unlikely, since we showed that denatured DNA transforms sevenfold less efficiently than native DNA. The cellular localization of the restriction enzymes remains unknown. It might be that transient alteration of these enzymes could result from a rise in temperature due to the Joule heating effect

at high voltages. Indeed, in *E. coli*, inhibition of restriction by a temperature shock was observed by Schell & Glover (1966). However, this observation cannot be extended to other corynebacteria, since Bonamy *et al.* (1990) found that restriction was not inactivated by electrotransformation in *Corynebacterium glutamicum* and *Corynebacterium melassecola*. Restriction systems are inhibited by electroporation in *Streptococcus pneumoniae* (Bonnassie *et al.*, 1989) and *Lactococcus lactis* (Langella & Chopin, 1989). It is possible that the inhibition of restriction by electroporation depends on the restriction system itself.

In conclusion, electrotransformation could be a useful method for research in genetic engineering of *B. lactofermentum*.

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