SHORT COMMUNICATION

An Efficient Method for the Introduction of Viral DNA into *Brevibacterium lactofermentum* Protoplasts

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A method for the introduction of a bacteriophage DNA into *Brevibacterium lactofermentum* protoplasts is described. Frequencies of 10^5 infective centres per μg DNA were easily achieved, the relationship between the number of infective centres and the amount of DNA being linear up to $5\ \mu\text{g}$ DNA per assay. This method can be used to introduce foreign DNA into these bacteria.

INTRODUCTION

Coryneform bacteria are a group of Gram-positive micro-organisms widely used for the industrial production of amino acids, mainly glutamic acid and lysine (Kinoshita, 1959). However, little is known about their genetics or the molecular basis of the regulation of their amino acid metabolism.

Unlike other species of Gram-positive bacteria, such as *Streptococcus* and *Bacillus* (Smith *et al.*, 1981), no natural transformation has been described for *Corynebacteriaceae*. Recently, a transformation system for *Brevibacterium lactofermentum* protoplasts was described (Santamaria *et al.*, 1985). We have used a transfection assay to develop an efficient system of introducing viral DNA into *B. lactofermentum*, based on the polyethylene glycol (PEG)- Ca^{2+} induced uptake of DNA by protoplasts of *Bacillus subtilis* (Chang & Cohen, 1978). The transfection procedure is discussed and compared with transformation.

METHODS

Bacteriophage techniques. Bacteriophage BL1 is a 14 kb circular double-stranded DNA phage isolated in our laboratory from a glutamic acid producer strain of *B. lactofermentum*. *B. lactofermentum* ATCC 13869 was used as a host for the maintenance and propagation of the phage. Phage particles were prepared using standard bacteriophage techniques and phage DNA was isolated by proteinase K treatment and phenol extraction of concentrated bacteriophage suspensions, as described for *B. subtilis* phage $\phi 29$ DNA (Inciarte *et al.*, 1976). Bacteriophage BL1 assays were at 30°C .

Formation and regeneration of protoplasts. *B. lactofermentum* cultures were grown at 30°C to $2\text{--}3 \times 10^8$ c.f.u. ml^{-1} in minimal medium (Kaneko & Sakaguchi, 1979) supplemented with 0.1% yeast extract (Y medium), pH 7.3. Cells were then treated with $0.18\ \mu\text{g}$ penicillin G ml^{-1} for 90 min as described by Kaneko & Sakaguchi (1979), harvested, washed in SMMY solution [four parts of $2 \times$ SMM buffer ($1 \times$ SMM is 0.5 M-sucrose, 0.02 M-maleate, 0.02 M-MgCl₂, pH 6.5; Chang & Cohen, 1978) and six parts of Y medium, pH 6.5] and finally resuspended in one-third the original volume of SMMY. Lysozyme (Sigma) was added to a final concentration of 1, 5 or $10\ \text{mg}\ \text{ml}^{-1}$ and the mixture was incubated at 30°C . At the times indicated (Table 1) samples were removed and protoplasts and intact cells were pelleted at $2600\ g$ for 15 min, washed and finally resuspended in SMMY. Appropriate dilutions were plated onto LB plates (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl) (viable cell count in this medium represents the population of non-protoplast cells) and onto DM3 regeneration medium (0.5% Casamino acids, 0.5 M-sodium succinate pH 7.3, 0.35% K₂HPO₄, 0.15%

Table 1. *Determination of optimal conditions of lysozyme for protoplast formation and regeneration*

Samples of cells (6×10^8 c.f.u. ml⁻¹) were incubated with lysozyme at the concentrations given and at different times samples were removed and plated onto LB plates (non-protoplast cells) and onto DM3 protoplast regeneration medium.

Incubation time (h)	Lysozyme concn (mg ml ⁻¹)	Formation*	Percentage regeneration†
1	1	1.5×10^{-2}	3
	5	3.3×10^{-4}	13
	10	1.6×10^{-5}	13.8
2	1	10^{-3}	4.6
	5	1.6×10^{-6}	15.8
	10	$< 1.6 \times 10^{-6}$	16.5
3	1	1.6×10^{-5}	8
	5	$< 1.6 \times 10^{-7}$	10
	10	$< 1.6 \times 10^{-7}$	12.5
4	1	8.3×10^{-7}	7.6
	5	ND	12.3
	10	1.6×10^{-8}	16.3

* Formation is the ratio between the viable cell count in LB plates after and before the lysozyme treatment; ND, not determined.

† Calculated as (no. of regenerated protoplasts/initial cell count) $\times$ 100. Number of regenerated protoplasts is DM3 viable count minus LB viable count.

KH₂PO₄, 0.5% yeast extract, 0.5% glucose, 20 mM-MgCl₂, 0.01% bovine serum albumin, 0.8% Bacto-agar; Chang & Cohen, 1978) in which colonies of regenerated protoplasts appeared after 4–5 d incubation at 30 °C.

Transfection. Phage DNA samples (up to 5 µg in 20 µl or less of 10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0; TE) were mixed with an equal volume of 2 $\times$ SMM and added to 0.5 ml of protoplast suspension (5×10^7 viable protoplasts). The suspension was then gently mixed with 1.5 ml 40% (w/v) PEG 6000 in 30 mM-CaCl₂ and incubated for 5 min at room temperature. PEG was then diluted by addition of 5 ml SMMY and the transfected protoplasts were harvested by centrifugation (2600 g, 10 min), resuspended in SMMY and incubated for 30 min at 30 °C to allow viral functions to be expressed. Samples (0.1 ml) of appropriate dilutions were mixed with 0.1 ml of an exponential culture of *B. lactofermentum* and 2.5 ml soft (0.6% agar) regeneration medium at 48 °C and plated onto regeneration medium DM4. This is the same as DM3 but contains 0.5 M-sucrose instead of the 0.5 M-sodium succinate that inhibits phage growth. Plates were incubated at 30 °C overnight and scored for the appearance of plaques.

To avoid an over-estimation of the frequency of transfected cells, it was essential to ensure that infective centres and not extracellular phages were scored. Phage titre in the resuspended protoplast pellet remained constant for at least 1 h after transfection and until this time no extracellular phage was detected. As protoplasts were plated after 30 min incubation after transfection, it was reasonable to assume that only infective centres were being scored in the assay.

RESULTS AND DISCUSSION

A critical factor for an efficient transfection/transformation method based on PEG-Ca²⁺ DNA uptake is a high yield of viable protoplasts. We have used a lysozyme treatment for the formation of *B. lactofermentum* protoplasts and DM3 medium (Chang & Cohen, 1978) for their regeneration. The results of a complete formation and regeneration experiment (which are in full agreement with those obtained from several experiments in which only one of the two variables was tested) are shown in Table 1. Protoplast formation (see footnote to Table 1) increased with time and lysozyme concentration. This latter factor affected the regeneration of protoplasts which, in two independent experiments, was optimal at 10 mg ml⁻¹, the highest lysozyme concentration tested (Table 1). After 2 h incubation with this concentration of lysozyme, protoplast formation was good and regeneration reached the highest level obtained (16.5%; Table 1). These conditions for protoplast formation represent a considerable shortening

Fig. 1. Samples of an exponentially growing culture of *B. lactofermentum* were collected at different times of growth (appropriate volume adjustments were made in order to use equivalent initial cell counts), and protoplasts were prepared and transfected with 1 μg BL1 DNA. Each sample was assayed for viable cell count (□) before the protoplast-forming treatment and for infective centres after the transfection assay (●). Growth was monitored by measuring OD₅₉₀ (○). Cell density of the culture corresponding to maximum transfection activity was 2.5×10^8 cells ml^{-1} .

of time over published procedures (Kaneko & Sakaguchi, 1978; Santamaría *et al.*, 1985) and were used for further experiments, with very reproducible results for protoplast formation (10^{-6} – 10^{-7}) and regeneration (around 10–20%). Santamaría *et al.* (1984) described a regeneration medium for *B. lactofermentum* that allowed protoplast regeneration in 4 d. We have used the standard DM3 medium used for *B. subtilis* in which protoplasts also regenerated in 4 d. For plating transfected protoplasts, however, sodium succinate has to be substituted by sucrose as the former inhibits phage growth.

Using this transfection assay we have established the factors affecting the uptake of viral DNA by *B. lactofermentum* protoplasts. As in plasmid DNA uptake (Santamaría *et al.*, 1985) the introduction of phage DNA was totally dependent on the PEG treatment, since in its absence no infective centres could be detected. PEG concentrations ranging from 15 to 30% were equally effective in inducing the DNA uptake with an efficiency of more than 10^5 p.f.u. μg^{-1} . A PEG concentration of 7.5% (w/v) resulted in a 1000-fold decrease in transfection efficiency. Very similar data were obtained in two different experiments. Although the transfection efficiency was similar when the PEG treatment was made in water, SMM medium or 30 mM- CaCl_2 , the highest score of infective centres was obtained in two different experiments when 30 mM- CaCl_2 was used. For this reason, we have routinely used 30% PEG 6000 in 30 mM- CaCl_2 . About 15% of the viable protoplasts regenerated after this PEG- Ca^{2+} treatment.

The physiological state of the culture before protoplast formation was important for protoplast transfection (Fig. 1). Samples containing equivalent cell counts were taken at different times of growth and protoplasts were made and transfected. The frequency of transfection showed a peak in the mid-exponential phase, corresponding to 2.5×10^8 c.f.u. ml^{-1} and decreased abruptly when the population reached the late exponential phase. This was not due to better protoplast formation since this factor was not significantly different between different samples.

This fact might represent the inability of cells to replicate the phage DNA efficiently or the presence of some specific factor needed for the DNA uptake that could be dependent on the growth of the culture. In the published procedure for plasmid transformation the culture is grown up to 1×10^8 c.f.u. ml⁻¹ (Santamaría *et al.*, 1985). In view of the results presented in Fig. 1, the growth of the culture should be carefully controlled to obtain a maximum efficiency of DNA uptake.

We have found a linear relationship between the amount of DNA and the transfection frequency, using quantities of input DNA ranging from 5 ng to 5 µg. In contrast with the results described with plasmid transformation, in which efficiency per µg dropped above 10 ng input DNA (Santamaría *et al.*, 1985), there were no dramatic differences in transfection efficiency using low (5 ng) or high (5 µg) amounts of transfecting DNA. With the optimized conditions we routinely obtained more than 1×10^5 p.f.u. per µg of phage DNA with a frequency of transfection per regenerated protoplast of 10^{-1} – 10^{-2} .

Although these data are notably lower than those described for *B. subtilis*, in which up to 40% transformants have been obtained using plasmid DNA (Chang & Cohen, 1978), it should be kept in mind that the transfection frequencies that were originally obtained in Ca²⁺-treated *Escherichia coli* cells were very similar (Mandel & Higa, 1970) and allowed the establishment of phage libraries.

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