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Effect of Ca^{2+} Ions on Plasmid Transformation of *Streptococcus lactis* Protoplasts

ATTE VON WRIGHT,* ANNA-MAIJA TAIMISTO, AND SEPPO SIVELÄ

Research and Development Department, VALIO Finnish Co-operative Dairies' Association, SF-00181 Helsinki, Finland

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The effects of Mg^{2+} and Ca^{2+} ions on the efficiency of the plasmid transformation of lysozyme-treated *Streptococcus lactis* protoplasts were compared. A 33-megadalton plasmid, pLP712, coding for lactose fermentation and a 6.5-megadalton plasmid, pGB301, coding for erythromycin and chloramphenicol resistance were used as model plasmids, and *S. lactis* MG1614 was the recipient. Replacing Mg^{2+} with Ca^{2+} in the transformation buffer was found to increase transformant frequency more than 10-fold with both plasmids.

An efficient protoplast transformation technique is essential for the genetic manipulation of lactic streptococci. Kondo and McKay reported successful transformation of mutanolysin-treated *Streptococcus lactis* protoplasts (6, 7) by the transformation protocol of Chang and Cohen (3). Using a 6.5-megadalton erythromycin-chloramphenicol-resistance plasmid, pGB301 (2), and a 20-min-polyethylene glycol (PEG) treatment time, they obtained transformation frequencies as high as 4×10^4 transformants per μg of DNA. Our aim was to optimize the transformation technique for lysozyme-treated *S. lactis* protoplasts. As the method of Kondo and McKay proved to be inefficient with our strains, we tried different protoplast concentrations and PEG treatment times, and, finally, we changed the ionic composition of the transformation mixture. We found that replacing Mg^{2+} with Ca^{2+} had a marked effect on the number of transformants obtained.

The *S. lactis* strains used were MG1299, MG1614, and VS118. MG1299 and MG1614, both derivatives of *S. lactis* NCDO712 (5), were kindly donated by M. Gasson, National Institute for Research in Dairying, Reading, England. MG1299 harbors the 33-megadalton plasmid, pLP712, coding for lactose fermentation and proteolysis (5). MG1614 is a plasmid-free strain with rifampin and streptomycin resistances as chromosomal markers. VS118 is a pGB301-carrying transformant of MG1614 constructed by us using DNA kindly donated by L. McKay, St. Paul, University of Minnesota.

M17 medium (9) supplemented with 0.5% glucose (GM17 for strains MG1614 and VS118) or lactose (LM17 for MG1299) was routinely used as the growth medium. Sucrose (0.4 M) was used in the medium as the osmotic stabilizer when protoplasts were handled. The selection medium for transformation experiments was LM17-sucrose agar (with 0.5 mg of bromcresol purple per liter as an indicator) for lactose fermentation and GM17-sucrose agar containing either erythromycin (2.5 μg/ml) or chloramphenicol (5 μg/ml) for antibiotic resistance. The cells were routinely grown at 30°C.

Plasmid DNA for transformation experiments was isolated from strains MG1299 and VS118 by the method of Anderson and McKay (1) and purified by CsCl-ethidium bromide gradient ultracentrifugation. The method of Anderson and McKay was also used for plasmid screening.

Protoplasting of the recipient strain, MG1614, was done by the method of Gasson (4), except that the lysozyme treatment was for 2 h at 37°C in GM17-sucrose medium (this modification was suggested by M. Gasson in a personal communication). For protoplasting, 100 ml of fresh medium was inoculated with 1 ml of an overnight culture of MG1614 and grown to an optical density at 600 nm of 0.5. The cells were harvested and suspended in an equal volume of protoplasting medium containing 4 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml. After treatment, the cells were centrifuged down at 4,000 rpm for 4 min in a bench-top centrifuge (CF-510-A; Labsystems, Helsinki, Finland) and washed once with either SMM buffer (0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$ [pH 6.5]) or SMC buffer (sucrose-maleate buffer with 0.05 M $CaCl_2$ [pH 6.5]), depending on the transformation procedure tested. Finally, the protoplasts were concentrated 10-fold in either SMM or SMC. The efficiency of protoplasting was checked by plating the cells on GM17 agar with or without sucrose. The number of osmotically sensitive protoplasts in the final suspension was fairly constant in each experiment (about 10^9/ml), and the fraction of osmotically resistant cells varied from 10 to 20% of the total.

The transformation buffer was SMM or SMC, depending on the experiment. Either buffer was also used as a base for the 30% PEG (PEG 4000; Fluka AG, Chemische Fabrik, Buchs, Switzerland) solution used in the experiments. For each transformation, 2 to 5 μl of plasmid DNA solution (corresponding to 1 μg of DNA) was mixed in an Eppendorf tube with an equal volume of double-strength transformation buffer. Various amounts of protoplast suspension (10 to 150 μl) were then added, followed immediately by 3 volumes of PEG solution. The PEG treatment was stopped after different periods (2 min to 2.5 h) by filling the tubes to the brim with transformation buffer. Finally, the cells were harvested by a 2-min centrifugation in an Osterode Haemofuge (Hereus Christ, Federal Republic of Germany). The protoplast pellet was suspended either in 0.5 ml of SMM or SMC (in the case of pLP712 transformation) or (with pGB301) in 1 ml of GM17-sucrose medium. In the latter case, the cells were incubated at room temperature for 1 h before plating for the expression of antibiotic resistance. The plating was done by the soft-agar-overlay technique. The plates were incubated at 30°C either for 48 h (lactose fermentation) or 96 h (antibiotic resistance).

Although the importance of Ca^{2+} ions in the plasmid transformation of gram-negative bacteria is well known (for a review, see reference 8), the role of divalent cations in the

* Corresponding author.

protoplast transformation of gram-positive bacteria has not been studied so extensively. However, according to our results, Ca^{2+} ions can have a marked affect on both the transformant frequency and the optimal PEG treatment time with *S. lactis* protoplasts. With SMC, the transformant frequencies with both pLP712 and pGB301 were 10- to 30-fold higher than those obtained with SMM (Fig. 1). The respective PEG treatment times were 2 to 5 min with $CaCl_2$ and 2 h with $MgCl_2$ (Fig. 2). It can also be seen (Fig. 1) that with SMC the transformant numbers were far less dependent on the optimal protoplast number in the transformation mixture than with SMM.

The transformant numbers obtained with pLP712 (about 2 $\times 10^4$ transformants per μg of DNA) were severalfold higher than those reported previously by Kondo and McKay for the lactose-fermentation plasmid of similar size (7). With pGB301, our transformant numbers, although higher than with pLP712, were within the same range as that reported by Kondo and McKay, i.e., 2 to 4 $\times$ 10^4/μg of DNA. In their transformation experiments, they used erythromycin selection, which in our system gave transformant numbers two orders of magnitude lower than those obtained with chloramphenicol selection (data not shown). As all our chloramphenicol-resistant transformants tested were also erythromycin resistant, and vice versa, and a plasmid similar in size to pGB301 was present in all the transformants screened, we conclude that the expression of erythromycin resistance, at least without induction with subinhibitory antibiotic concentrations, was low in our recipient strain. This might indicate that the expression of chloramphenicol resistance was less than optimal, and this may have caused the transformant yields to be lower than would be expected on the basis of plasmid size.

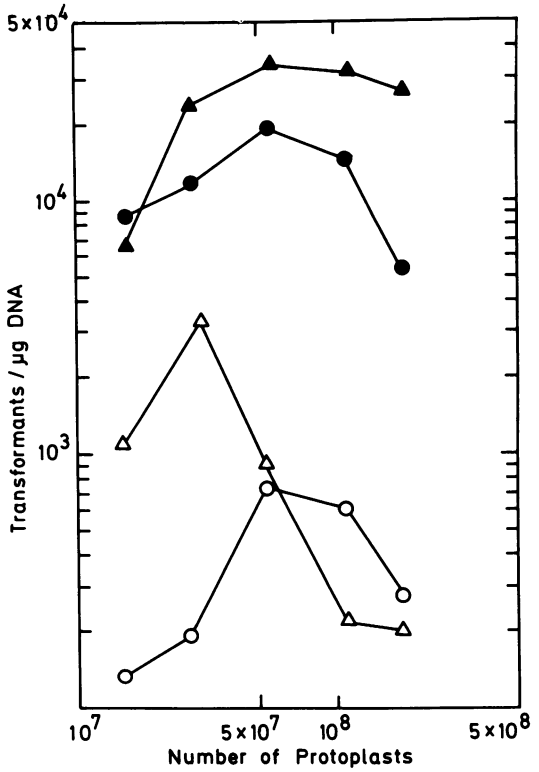

FIG. 1. Efficiency of plasmid transformation in the presence of Ca^{2+} (closed symbols) or Mg^{2+} (open symbols) ions in the transformation mixture. Symbols: ● and ○, pLP712; ▲ and △, pGB301. The duration of PEG treatment was 1 h, and 1 μg of plasmid DNA was used. Chloramphenicol was used as the selective agent with pGB301.

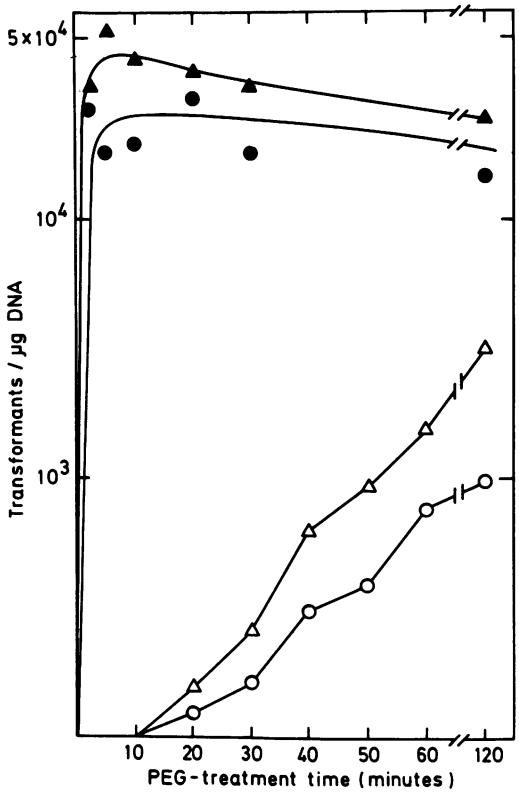

FIG. 2. Effect of Ca^{2+} (closed symbols) or Mg^{2+} (open symbols) ions on the optimal PEG treatment time for plasmid transformation. Symbols: ● and ○, pLP712; ▲ and △, pGB301. The concentration of plasmid DNA in the total transformation mixture was 1 μg. Chloramphenicol was used as the selective agent with pGB301.

At this stage, we can only speculate about the role of Ca^{2+} ions in the transformation process. It is possible that Ca^{2+} somehow makes the cell membrane more permeable to DNA or, alternatively, that the conformation of DNA is more suitable for transformation in the presence of Ca^{2+} ions than with Mg^{2+}. Another explanation for the results obtained could be the presence of some protoplast-associated nuclease requiring Mg^{2+} as a cofactor.

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