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High-Frequency Transformation, by Electroporation, of *Lactococcus lactis* subsp. *cremoris* Grown with Glycine in Osmotically Stabilized Media

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An efficient method for genetic transformation of lactococci by electroporation is presented. Highly competent lactococci for electrotransformation were obtained by growing cells in media containing high concentrations of glycine and 0.5 M sucrose as the osmotic stabilizers. These cells could be stored at −85°C without loss of competence. With *Lactococcus lactis* subsp. *cremoris* BC101, a transformation frequency of 5.7 × 10^7 transformants per μg of pIL253 DNA was obtained, which represents 5% of the surviving cells. All the lactococcal strains tested could be transformed by the present method.

Strains of *Lactococcus lactis* subsp. *cremoris* are important starter organisms in the production of cheese. Strain improvement by modern gene technology as well as genetic studies of these organisms has been hampered by the lack of efficient genetic transformation systems. For the closely related *L. lactis* subsp. *lactis*, successful transformation procedures have been developed. The highest transformation frequency reported was 5 × 10^6 erythromycin-resistant transformants per μg of pIL204 DNA (16). Polyethylene glycol-mediated protoplast transformation was used in this work. However, this method is not effective for all strains and has been shown to work for only a few *L. lactis* subsp. *cremoris* strains with transformation frequencies of about 10^3/μg of DNA or less (15, 17).

Transformation by electroporation is a recent method that has been successfully employed with a number of bacterial species (8; for a review, see reference 2). This technique is less tedious and time consuming than protoplast transformation and has proved useful in species previously regarded as untransformable. Harlander (6) was the first to transform *L. lactis* subsp. *lactis* by electroporation. Extended work by McIntyre and Harlander (10) did not improve the transformation frequency significantly.

Powell et al. (13) were able to transform several strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* by electroporation, but the transformation frequencies obtained were generally very low, and not all of the strains tested could be transformed by their procedure. However, a high transformation frequency was obtained with *L. lactis* subsp. *lactis* LM0230 treated with lysozyme before electroporation, indicating that the cell wall is a physical barrier to the entering DNA. This notion was supported by the work of van der Lelie et al. (17), who incorporated 40 mM DL-threonine in the growth medium to obtain cells with weakened cell walls. These cells could be transformed by electroporation, but transformation frequencies were still quite low. Transformants were not obtained when threonine was omitted from the growth medium.

Our initial attempts to use these two procedures (13, 17) in transforming *L. lactis* subsp. *cremoris* BC101 were unsuccessful. The published data suggested that this could result from inadequate weakening of the cell wall; the data of Powell et al. (13) indicate that most of the cells able to take up DNA are osmotically fragile. Such cells cannot be obtained by using cell wall-weakening agents in hypotonic growth media. In the work presented here, the cells were grown in osmotically stabilized media containing high concentrations of glycine as an inhibitor of cell wall formation. These cells were transformed by electroporation at high frequencies.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. lactis* subsp. *cremoris* BC101 and *L. lactis* subsp. *lactis* LM2336 (both plasmid-free, Lac⁻) were kindly provided by T. Langsrud, Agricultural University of Norway, Ås, Norway. *L. lactis* subsp. *cremoris* GS was isolated from a commercial starter culture (C. Hansen, Copenhagen, Denmark). *L. lactis* subsp. *lactis* IL1837(pIL253) was kindly provided by A. Chopin, Institut National de la Recherche Agronomique, Jouy-en-Josas, France. Other lactococcal strains used in this study were from The National Collection of Food Bacteria (Reading, United Kingdom). All lactococcal strains were grown at 30°C in M17 supplemented with 0.5% glucose (GM17) unless otherwise stated. *E. coli* JM109(pSA3) was kindly provided by J. J. Ferretti, University of Oklahoma Health Science Center, Oklahoma City.

Transformation protocol. To obtain competent cells, the cultures were grown to an optical density at 600 nm of 0.5 to 0.8 and then diluted 100-fold in SGM17 (GM17 containing 0.5 M sucrose) supplemented with glycine as indicated in the text. After growth at 30°C to an optical density at 600 nm of 0.2 to 0.7, the cells were harvested by centrifugation at 4°C at 5,000 × *g*. Following two washes in ice-cold 0.5 M sucrose containing 10% glycerol, the cells were suspended in 1/100 culture volume of washing solution and then stored in aliquots at −85°C until use.

The cell suspensions were thawed on ice. Portions (40 μl) were mixed with 1 μl of DNA dissolved in 10 mM Tris hydrochloride–1 mM EDTA (pH 7.5) and then transferred to an ice-cooled electroporation cuvette (2-mm electrode gap) and exposed to a single electrical pulse. The pulse was delivered by a Gene-Pulser (Bio-Rad Laboratories, Richmond, Calif.) set at 25 μF and normally at 2.0 kV. The

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cuvette was connected in parallel to a 200-Ω resistor (pulse controller; Bio-Rad), resulting in time constants of 4.5 to 5 ms. Immediately following the discharge, the suspensions were mixed with 0.96 ml of ice-cold SGM17 containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ (SGM17MC) and left on ice for about 5 min. Appropriate dilutions were then made in SGM17MC, and the cells were incubated at 30°C for 2 h before 100-μl portions were spread on selective streptococcal regeneration medium (SR) plates (12) containing (per liter) 10 g of tryptone, 5 g of yeast extract, 200 g of sucrose, 10 g of glucose, 25 g of gelatin, 15 g of agar, 2.5 mM $MgCl_2$, and 2.5 mM $CaCl_2$ (pH 6.8). The plates contained 1 μg of erythromycin per ml for the selection of erythromycin-resistant transformants. Transformants were enumerated after 2 days of incubation at 30°C.

DNA isolation and analysis. Erythromycin-resistant transformants were grown in GM17 containing 10 μg of erythromycin per ml. Plasmids were isolated by the alkaline lysis procedure described by Klaenhammer (7) and analyzed by agarose gel electrophoresis as described by Maniatis et al. (9). The plasmids were transferred from agarose gels to Hybond-N (Amersham International, Amersham, United Kingdom) nylon filters by vacuum transfer and then subjected to Southern analysis according to Maniatis et al. (9). The plasmids used as probes were purified by CsCl equilibrium centrifugation before being ^{32}P-labeled by nick translation. DNA concentrations were measured fluorometrically, by using a TKO Mini Fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.); the Hoechst fluorochrome dye 33258 (Polysciences Inc., Warrington, Pa.), which binds specific for DNA, was also used.

RESULTS

Optimization of transformation of *L. lactis* subsp. *cremoris* BC101 by electroporation. By modifying the procedure of Dower et al. (4) for transformation of *Escherichia coli*, we were able to transform strain BC101 by electroporation. The cells were transformed with pIL253, a cloning vector derived from pAMβ1 that has been constructed for cloning in gram-positive organisms (3). To increase transformation frequency, glycine was incorporated in the growth medium of strain BC101 to weaken its cell wall. The effect of glycine on growth is shown in Fig. 1. The presence of sucrose itself will inhibit growth of strain BC101. In the absence of sucrose, no growth was observed with more than 2% glycine in the medium, whereas 0.5 M sucrose-containing medium supported growth in up to 3% glycine. Consequently, the cells without sucrose were relatively more sensitive to growth inhibition by glycine than were cells grown in the presence of an osmotic stabilizer (Fig. 1). This suggested that growth inhibition caused by glycine was a result of its effect on cell wall formation. Cells from stationary-phase cultures were less sensitive to glycine and could grow with 4% glycine in SGM17. Microscopic examination revealed swollen cells, spheroplasts, and some ghosts from cultures grown at the highest glycine concentrations in SGM17. This was not observed in cultures grown without sucrose.

Figure 1 also shows the effect of glycine on the transformability of strain BC101. Adding glycine to the growth medium resulted in an increase in transformability. The highest transformation frequencies were obtained with cells grown at the highest glycine concentrations. For cells grown in SGM17, transformation frequency increased exponentially with respect to glycine concentration in the range of 0.5 to 2%. The effect of glycine was much less pronounced without sucrose. Thus, sucrose caused an increase in transformability even at glycine concentrations that the cells could tolerate without osmotic protection.

Figure 2 shows the effect of electrical field strength on the

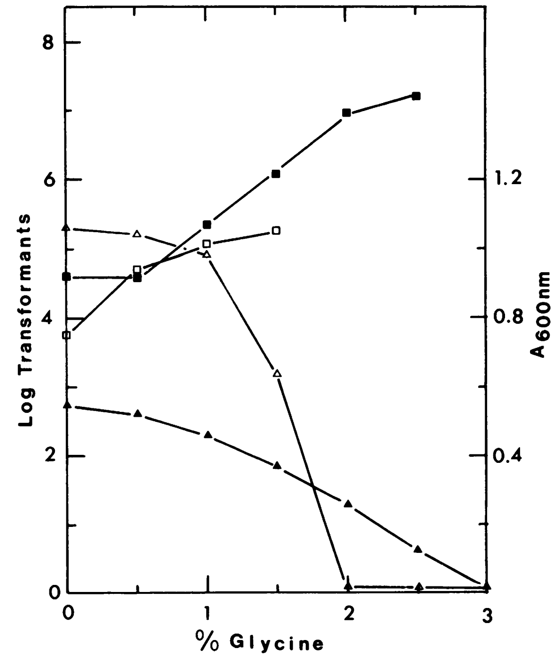

FIG. 1. Effect of glycine on growth and plasmid transformation of *L. lactis* subsp. *cremoris* BC101 with and without 0.5 M sucrose in the growth medium. The cells were grown overnight. Symbols: □ and △, growth in the presence and absence of sucrose, respectively; ■ and ▲, transformation in the presence and absence of sucrose, respectively.

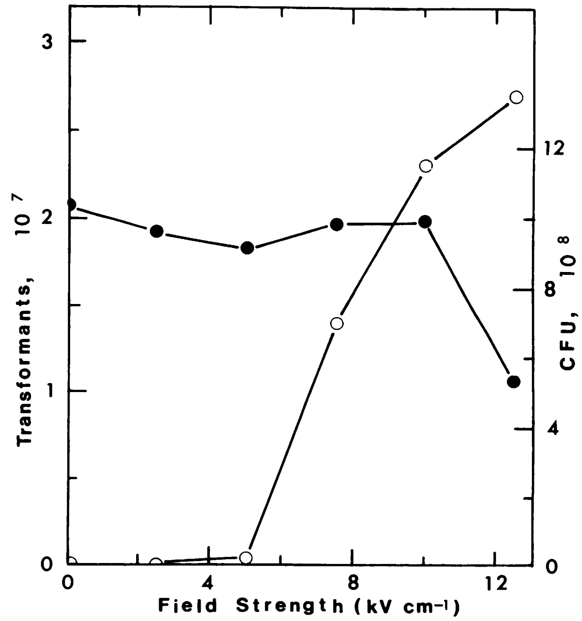

FIG. 2. Effect of electrical field strength on transformation efficiency (○) and survival (●) of *L. lactis* subsp. *cremoris* BC101. The cells were grown with 3% glycine in SGM17.

transformation of strain BC101. High transformation frequencies were observed at 5 kV/cm and higher. At 2.5 kV/cm, only 50 transformants per 0.8 μg of DNA were obtained. The highest transformation frequency, 2.7×10^7 transformants per 0.8 μg in this experiment, was found at 12.5 kV/cm, which is the maximum field strength for the equipment used here. The number of transformants was only slightly lower at 10 kV/cm. Since there was a greater possibility of arcing when 12.5 kV/cm was applied, electroporation was normally performed at 10 kV/cm.

At field strengths up to 10 kV/cm, nearly 100% of the cells survived the pulse, as judged by plate counts. In the experiment shown in Fig. 2, 5% of the survivors were transformed with pIL253 at 12.5 kV/cm.

The lactococci exist in chains of various lengths depending on the individual strains and growth conditions. Microscopic analysis did not indicate that chain shortening occurred as a result of the electrical discharge.

Storing competent strain BC101 cells at −85°C in 0.5 M sucrose or 0.5 M sucrose containing 10% glycerol did not affect their transformability. At high cell densities and high voltages, the presence of 10% glycerol caused an increase in time constant and reduced the risk of arcing. Consequently, glycerol was added to the cell suspensions before electroporation and, for simplicity, it was included in the medium used for freezing the cells.

At 10 kV/cm, the number of transformants was proportional to the amount of DNA used for transformation in the range of 0.8 ng to 0.8 μg. A linear relationship between transformation frequency and cell density in the range of 2×10^9 to 5×10^{10} CFU/ml was also found.

Strain BC101 was also transformed with pSA3 isolated from *E. coli* JM109 and from strain BC101 itself at frequencies of 5×10^3 and 2×10^7/μg of DNA, respectively, suggesting the presence of a restriction barrier for the transformation with DNA isolated from *E. coli* JM109.

Furthermore, pIL253 was digested with *Bam*HI or *Eco*RI, and the linearized plasmid was treated with T4 DNA ligase and transformed in strain BC101 at frequencies of 10 to 50% of that of the native plasmid. When the ligase step was omitted, the frequencies were about 1,000-fold lower. This experiment showed that the treatments commonly involved in cloning do not affect this transformation procedure.

According to van der Lelie et al. (17), SR is superior to SGM17 for regenerating protoplasts of most strains of *L. lactis* subsp. *cremoris*. We obtained the highest transformation frequencies by using SR instead of SGM17 or GM17 for the recovery of transformants. With strain BC101, about three times as many transformants were recovered on SR as on GM17, and transformants were visible after 15 h of incubation at 30°C. Growth on SGM17 plates was slower.

Transformation of other lactococcal strains. The method used to obtain high-frequency transformation of strain BC101 was applied to several other lactococcal strains. Most strains were more sensitive to growth inhibition by glycine than was strain BC101, and the presence of sucrose apparently did not increase glycine tolerance in all strains, as judged by 24 h of growth. However, swollen cells were observed in all strains grown with the medium supplements listed in Table 1. In most of the strains tested, 0.5 M sucrose could be replaced by 0.25 M sodium succinate as the osmotic stabilizer. In general, growth was faster with sucrose than with succinate, and it was therefore preferred. In the GS strain, however, higher transformation frequencies were obtained with cells grown with succinate than with those grown with sucrose. *L. lactis* subsp. *cremoris* NCDO 607 (type strain) could grow with 2.5% glycine in GM17, but in the presence of sucrose, about 1% glycine was the upper limit for growth. In the presence of 0.25 M sodium succinate, the glycine tolerance of strain NCDO 607 was not reduced.

TABLE 1. Transformation of *L. lactis* subsp. *cremoris* strains by electroporation[a]

Strain	% Glycine in growth medium	Transformants/μg of pIL253 DNA
NCDO 495	1	1.7×10^6
NCDO 504	1	2.0×10^4
NCDO 607	1	5.9×10^5
NCDO 893	1	2.1×10^5
NCDO 924	1	6.3×10^3
NCDO 1986	1	5.0×10^3
NCDO 1997	1	2.8×10^5
NCDO 2004	1	2.2×10^3
GS	0.3[b]	2.6×10^5
BC101	3	5.7×10^7
LM2336[c]	4	1.4×10^7

[a] All strains were electroporated at 10 kV/cm. Frozen cells were used.

[b] 0.25 M sodium succinate was used as osmotic stabilizer.

[c] *L. lactis* subsp. *lactis*.

All the strains tested were transformed with pIL253, although at various efficiencies (Table 1). However, no further effort was made to optimize the conditions to improve the transformation efficiency of each strain. In several strains (NCDO 504, NCDO 924, NCDO 1986, and NCDO 1997), transformants could not be recovered when spread on GM17 plates instead of on SR. Except in the case of strain NCDO 1986, transformant colonies on SR plates could be transferred directly to GM17 broth. In strain NCDO 1986, the transformants were transferred to SGM17 broth and then to GM17.

Plasmid analysis of transformants. Transformation was verified by agarose gel electrophoresis and Southern DNA hybridization analysis of the plasmids from various transformants (Fig. 3). All transformants contained pIL253, while the control strains did not hybridize to the ^{32}P-labeled pIL253.

Plasmid pIL253 is a 4.8-kilobase plasmid in its monomeric form. In *L. lactis* subsp. *lactis* IL1837, dimers and trimers tended to be the dominant forms (Fig. 3). The pIL253 oligomers were identified by restriction enzyme analysis (data not shown).

The molecular weights of the oligomers were confirmed by agarose gel electrophoresis, by using supercoiled plasmids as standards. Results from limiting DNase I digest of pIL253 DNA in the presence of Mn^{2+} to create open circles and linear DNA (1) supported our conclusion that oligomers were made in the lactococci (data not presented).

In one plasmid preparation from *L. lactis* subsp. *lactis* IL1837, a deleted form of pIL253 (4.0 kilobases) was found. Restriction enzyme analysis suggested that the multiple cloning site was part of the deleted fragment of 0.8 kilobase (data not shown). The deleted form of pIL253 also transformed strain BC101 (Fig. 3A, lane 6).

DISCUSSION

This work describes a method for genetic transformation of *L. lactis* subsp. *cremoris*, for which efficient transformation systems have been lacking. Purification of the transforming plasmid by CsCl gradient centrifugation did not increase transformation frequency. This is in contrast to the finding of Sanders and Nicholson (14), who observed a

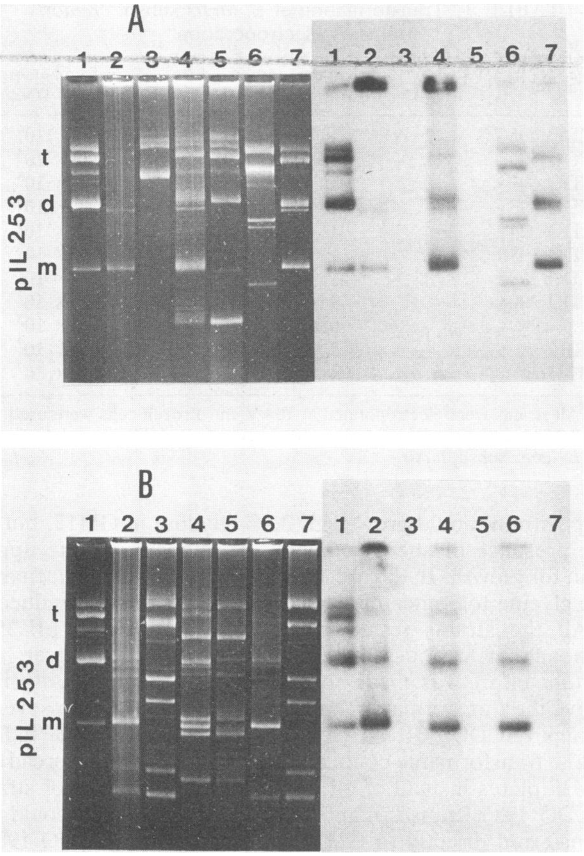

FIG. 3. Agarose gel electrophoresis and Southern blot analyses of plasmids isolated from various *L. lactis* subsp. *cremoris* strains before and after pIL253 transformation by using purified, nick-translated, ^{32}P-labeled pIL253 as a radioactive probe. Lanes 1 in panels A and B are controls of pIL253 isolated from *L. lactis* subsp. *lactis* IL1837. (A) Lanes: 2, transformed NCDO 1986; 3, wild-type NCDO 1896; 4, transformed NCDO 1997; 5, wild-type NCDO 1997; 6, strain BC101 transformed with a deleted form of pIL263; 7, transformed BC101. (B) Lanes: 2, transformed NCDO 504; 3, wild-type NCDO 504; 4, transformed NCDO 607; 5, wild-type NCDO 607; 6, transformed NCDO 924; 7, wild-type NCDO 924. m, d, and t indicate monomers, dimers, and trimers of pIL253, respectively.

10-fold increase in the transformation efficiency of pSA3 when purified by CsCl gradient centrifugation. The use of frozen cells and plasmid minipreparations makes the method described here efficient and time saving compared with the published procedures for the transformation of lactococci. Moreover, it is highly reproducible and seems general, since all the lactococcal strains tested were transformed.

The results of our work are in agreement with earlier findings that the rigid cell wall poses a barrier to DNA uptake by lactococci (13, 17). Usually, the cells are made competent by the use of lysozyme or mutanolysin, and a crucial step in transformation is the regeneration of the protoplasts or spheroplasts. This normally takes several days. By contrast, the restoration of wall integrity of cells made competent by the method used here is usually rapid; within 2 h, about one-third of the transformants of strain BC101 are able to grow in hypotonic media. This method to weaken the cell walls is also effective on strains like BC101 that are quite resistant to lysozyme and mutanolysin. We were not able to obtain swollen spheroplasts of this strain by using wall-digesting enzymes. Apparent resistance to these enzymes is quite widespread among lactococci (5, 18).

The use of a cell wall-weakening agent during growth may also be advantageous to the use of lytic enzymes in weakening the cell wall, since it ensures that the competent cells produced are viable. Furthermore, a certain wall strength seems to be necessary for the cells to survive electroporation. Powell et al. (13) showed that cells subjected to extensive lysozyme treatment ruptured on electroporation. This was not observed with the cells used in this study.

It is likely that the present method to obtain competent cells for electrotransformation is applicable to other gram-positive organisms. Incubation in sucrose itself is known to cause a weakening of the cell wall in several bacteria (11, 14), and this may explain the finding that in some strains, glycine tolerance was not enhanced by the presence of sucrose during growth. In most strains, however, this effect of sucrose did not appear to significantly reduce the recovery rate of transformants.

So far, this transformation procedure has been effective in the cloning of DNA fragments of a temperate *L. lactis* subsp. *cremoris* bacteriophage (D. Lillehaug, personal communication) in strain BC101. The fragments were cloned into the shuttle vector pSA3 in *E. coli*, and the recombinant plasmids were then transformed into BC101. Thus, recombinant DNA can be cloned directly in *L. lactis* subsp. *cremoris*.

Since a large portion of the survivors are transformed by the present method, the recovery of clones transformed with DNA not carrying a selectable marker gene should be possible. This may be of importance in the improvement of starter organisms by gene manipulation.

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