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Plasmid Transformation of *Streptococcus lactis* Protoplasts: Optimization and Use in Molecular Cloning†

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The parameters affecting polyethylene glycol-induced plasmid transformation of *Streptococcus lactis* LM0230 protoplasts were examined to increase the transformation frequency. In contrast to spreading protoplasts over the surface of an agar medium, their incorporation into soft agar overlays enhanced regeneration of protoplasts and eliminated variability in transformation frequencies. Polyethylene glycol with a molecular weight of 3,350 at a final concentration of 22.5% yielded optimal transformation. A 20-min polyethylene glycol treatment of protoplasts in the presence of DNA was necessary for maximal transformation. The number of transformants recovered increased as the protoplast and DNA concentration increased over a range of 3.0×10^6 to 3.0×10^8 protoplasts and 0.25 to 4.0 μg of DNA per assay, respectively. With these parameters, transformation was increased to 5×10^3 to 4×10^4 transformants per μg of DNA. Linear and recombinant plasmid DNA transformed, but at frequencies 10- to 100-fold lower than that of covalently closed circular DNA. Transformation of recombinant DNA molecules enabled the cloning of restriction endonuclease fragments coding for lactose metabolism into *S. lactis* LM0230 with the *Streptococcus sanguis* cloning vector, pGB301. These results demonstrated that the transformation frequency is sufficient to clone plasmid-coded genes which should prove useful for strain improvement of dairy starter cultures.

The group N streptococci, which include strains of *Streptococcus lactis*, *Streptococcus lactis* subsp. *diacetylactis*, and *Streptococcus cremoris*, are used extensively by the dairy industry for the production of fermented milk products. Recent development of gene transfer systems in these bacteria has increased our knowledge of their genetics and plasmid biology. Conjugation and transduction have now been well documented (see review by Gasson in reference 12), and transformation (19), transfection (13), and protoplast fusion (11) have been reported. Although these gene transfer mechanisms have aided in studying the genetics and plasmid biology of these organisms, the development of a more efficient plasmid transformation system is vital for further genetic studies and for the use of recombinant DNA technology for strain improvement.

Recently, we described the polyethylene glycol (PEG)-induced transformation of *S. lactis* protoplasts with plasmid DNA (19). Formation of protoplasts was essential for transformation, and we found that mutanolysin is effective in generating protoplasts of dairy streptococci (20). Although the use of lysozyme in the formation of protoplasts in lactic streptococci has been reported (11), lactic streptococci are fairly resistant to lysozyme. Using lysozyme and α-amylase, Okamoto et al. (28) showed that lysozyme concentration was critical for efficient protoplast formation and that a concentration of 30 μg of lysozyme per ml was effective in protoplast formation. Mutanolysin was used in the formation of protoplasts for transformation in our experiments, since the procedure takes only 20 min for complete protoplast formation in contrast to 3 h with lysozyme (11, 28).

Initial protoplast transformation frequencies with pLM2103, a 23-megadalton (Mdal) transductionally shortened plasmid coding for lactose utilization (10), were low (ca. 8.5 transformants per μg of DNA). In this study, we examined several parameters affecting protoplast transformation in an attempt to increase transformation frequencies. We also examined the feasibility of using this transformation system to clone plasmid-coded genes in *S. lactis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains and plasmids used in this study are listed in Table 1. Strains of *S. lactis* were maintained by biweekly transfer at 32°C in M17 broth (33) containing either 0.5% glucose (M17-G) or lactose (M17-L). *S. sanguis* Challis SM301 was propagated in brain heart infusion broth at 37°C. We obtained SM301 harboring pGB301 (3, 4) from Joseph Ferretti (Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City). Plasmid pGB301 was isolated from SM301 and transformed into *S. lactis* LM0230, a plasmid-cured derivative of *S. lactis* C2 (10). A resulting transformant, JK301, which contained pGB301 was used to isolate plasmid DNA for subsequent transformation and cloning experiments.

DNA preparations. Plasmid DNA was isolated by the method of Anderson and McKay (1). Plasmids used for transformation experiments, restriction endonuclease analysis, and molecular cloning experiments were further purified by cesium chloride-ethidium bromide density gradient centrifugation (18) followed by dialysis for 2 days against TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with several buffer changes. Plasmid DNA was ethanol precipitated and suspended in TE buffer. Plasmids were visualized by agarose gel electrophoresis on a tandem 14-cm, 0.6% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA, pH 8.0). Electrophoresis was for 6 h at 100 V. DNA concentration was determined spectrophotometrically (optical density at 260 nm/optical density at 280 nm) or by the gel method as described by Maniatis et al. (23).

Restriction endonuclease studies. All restiction enzymes were purchased from Bethesda Research Laboratories. Digestions were performed as described by Maniatis et al. (23) with lambda *Hin*dIII fragments used as mobility reference fragments. Restriction fragments were separated on a tan-

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TABLE 1. Bacterial strains and plasmids

Strain	Relevant plasmid	Plasmid sizes (Mdal)	Plasmid-conferred phenotype	Description (reference)
S. lactis ML3		33, 5.5, 2, 1	Lac^+	Parent (21)
S. lactis C20		29, 23, 2.2, 1.5	Lac^+	Parent (25)
S. lactis M18		45, 29, 25, 4.5, 1	Lac^+	Parent (21)
S. lactis C10		40, 25, 4.5, 1	Lac^+	Parent (21)
S. lactis LM0230	plasmid cured			Plasmid-cured, Lac^- derivative of *S. lactis* C2 (10)
S. lactis 2301β	pAMβ1	17	Em^r	Ery^r transconjugant of *S. faecalis* JH2-2 (8,17) × *S. lactis* LM2301 (this study)
S. lactis LM0232	pLM2001	24	Lac^+	(10)
S. lactis LM0231	pLM2103	23	Lac^+	(10)
S. lactis PN221	pPN221	33	Lac^+	(34)
S. lactis JK301	pGB301	6.5	Em^r	Em^r transformant (this study) of *S. lactis* LM0230
S. sanguis SM301	pGB301	6.5	Em^r	*S. sanguis* cloning vector (3, 4)

dem 14-cm horizontal agarose gel containing 0.6 or 0.7% agarose in TAE buffer. Electrophoresis was for 5 to 6 h at 80 V.

Protoplast formation. Formation of protoplasts was performed as described previously (19) with modifications. A 1% inoculum of an active 16-h M17-G broth culture was made into 30 ml of M17-G broth. Cells were grown for 2 h at 32°C, centrifuged at 4,500 × *g*, washed in cold (4°C) distilled water, and suspended in 7.6 ml of 0.5 M sucrose in 0.01 M Tris-hydrochloride (pH 7.0). Mutanolysin (Miles Scientific, Naperville, Ill.) (35, 36) was added to a final concentration of 25 μg/ml, and the cell suspension was incubated at 37°C for 20 min. The protoplast suspension was centrifuged at 2,600 × *g*, washed in 5 to 10 ml of SMMB buffer (0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$, 1% bovine serum albumin, pH 6.5), and resuspended in 1.0 ml of SMMB buffer.

Regeneration studies. *S. lactis* LM0230 was used as the test strain in all regeneration studies. The protoplast suspension was diluted with both SMMB buffer and distilled water so that counts between 30 and 300 CFU per plate were obtained. The number of osmotically stable cells, i.e., cells which have not formed protoplasts and thus are resistant to osmotic shock, was determined by dilution in distilled water and plating on M17-G agar. The number of protoplasts formed was determined by diluting protoplasts in SMMB buffer and plating on M17-G agar containing 0.5 M sucrose (SM17-G). This count minus the number of osmotically stable cells determines the number of viable protoplasts. The number of input bacteria was determined by omitting the mutanolysin treatment step. Bacterial counts were made by dilution in SMMB buffer and plating on SM17-G agar.

Initially, counts were determined by the spread plate technique. Later, cells were plated with soft agar overlays. Usually, 0.1 or 0.2 ml of an appropriate dilution of protoplasts was added to 3.0 ml of SM17-G top agar (0.5% agar), gently mixed, and overlaid on SM17-G bottom agar (1.5% agar) plates. Plates were incubated at 28°C for 5 to 7 days before counting. Colonies derived from protoplasts appeared after 72 h. Calculation of regeneration frequencies was as follows: % regeneration = [(protoplasts)/(input bacteria − osmotically stable cells)] × 100.

Transformation. Transformation experiments were performed essentially as described previously (19) but with modifications. Plasmid DNA (0.75 to 1.0 μg) in TE buffer was added to an equal volume of 2× SMM (19) buffer. DNA was then added to 0.5 ml of protoplast suspension, followed immediately by the addition of 1.5 ml of a 30% PEG solution (Sigma Chemical Co., St. Louis, Mo.; 30 g of PEG 3350, 50 ml of 2× SMM brought up to 100 ml with distilled water). The transformation mixture was allowed to incubate for at least 20 min at room temperature, and 5 ml of SMMB buffer was then added to dilute the PEG. Protoplasts were recovered by centrifugation at 2,600 × *g* and suspended in 0.5 ml of SMMB. A total of 0.4 ml of 2× SMM–M17G broth (1:1) was added, and cells were allowed to incubate for at least 30 min to allow for expression when selecting for antibiotic resistance.

Appropriate dilutions of the transformed cells were made in SMMB and then plated with soft agar overlays as described above. Erythromycin-resistant (Em^r) transformants were selected by supplementing SM17-G top and bottom agars with 5 μg of erythromycin per ml. Lactose-positive (Lac^+) transformants were detected by the addition of 40 mg of bromocresol purple per ml to SM17-L top and bottom agar. The concentration of β-glycerophosphate was decreased from 1.9 to 0.5% so that Lac^+ transformants could be adequately scored. Incubation of plates was at 28°C for 5 to 7 days before transformants were scored. Transformants began to appear after 72 h and reached a maximum after ca. 5 to 7 days. In all transformation experiments, incubation of protoplasts without DNA yielded no Em^r or Lac^+ colonies. No transformants were observed if the PEG treatment step was omitted. Addition of DNase I (Sigma) to plasmid preparations before transformation was previously shown to inhibit transformation (19). Protoplast concentration was kept constant for each transformation trial by preparing and pooling protoplast suspensions before distributing them for use in transformation. All data for transformation experiments were from two or more independent trials.

Molecular cloning. Plasmid pGB301, a 9.8-kilobase-pair (kb) *Streptococcus sanguis* cloning vector, was used to clone genes responsible for lactose utilization from pLM2001, a 23-Mdal *S. lactis* LM0232 plasmid coding for lactose utilization (10). Plasmids pLM2001 and pGB301 were separately digested to completion with the appropriate re-

TABLE 2. Effect of plating method and PEG treatment on regeneration frequency of *S. lactis* LM0230 protoplasts[a]

Treatment[a]	Expt no.	% Regeneration	
		Spread plates	Overlays
Without PEG	1	1.0	54.0
	2	1.0	12.0
With PEG	1	1.1	3.6
	2	0.9	3.2

[a] Protoplasts were regenerated with spread plates or soft agar overlays and treated with or without PEG. PEG treatment involved a 15-min incubation of protoplasts with PEG 3350 at a final concentration of 22.5%. Treated protoplasts were then harvested, suspended, and plated.

striction endonuclease, followed by phenol-chloroform extraction of the enzyme and ethanol precipitation of DNA. Passenger DNA was suspended in TE buffer, whereas vector DNA was suspended in 10 mM Tris-hydrochloride (pH 7.2) and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, Ind.) for 30 min at 37°C (10 U/μg of DNA). The enzyme was heat inactivated at 65°C for 10 min and removed by phenol-chloroform extraction. DNA was ethanol precipitated and suspended in TE buffer.

Passenger and vector DNA were mixed at 3:1 and 1:1 ratios for experiments 1 and 2, respectively. Ligation was performed as described by Maniatis et al. (23) in a volume of 100 μl with 1.0 and 3.0 μg of vector DNA in experiments 1 and 2, respectively. T4 DNA ligase (Biotec, Madison, Wis.) was added at 2 U/μg of DNA and incubated for 16 to 24 h at 16°C. Ligase was heat inactivated for 10 min at 65°C and removed by phenol-chloroform extraction. DNA was ethanol precipitated and suspended in 30 μl of TE buffer. Recombinant DNA molecules were then used in the transformation of *S. lactis* LM0230. DNA concentrations and ligation of DNA were estimated by agarose gel electrophoresis with a horizontal minigel. Electrophoresis was for 1.5 h at 60 V through a 0.8% agarose gel in TAE buffer.

Transformation was performed as described above. Transformant colonies were obtained by selection of the Em^r marker of the vector and then replica plated onto bromocresol purple-lactose indicator agar (26). All Lac$^+$ transformant clones were transferred to M17 broth for further analysis.

Analysis of insert fragments. Recombinant plasmid DNA was isolated from Lac$^+$ transformants, and cloned fragments were identified by restriction endonuclease digestion. If the fragments cloned could not be definitively identified, Southern blot hybridization was performed with a commercially available DNA-DNA blot hybridization system (Enzo Bio-Probe System; Enzo Biochemicals, Inc., New York, N.Y.). Hybridization was performed by transferring a restriction digestion of pLM2001 to nitrocellulose HAHY paper (Millipore Corp., Bedford, Mass.) by the Southern method (31), as modified by Maniatis et al. (23). A nick-translated biotinylated probe (22) of the recombinant plasmid was constructed according to the manufacturer's instructions. Hybridization of the probe to specific fragments was performed under stringent temperature conditions at 68°C. Detection of biotinylated probe hybridized to specific fragments by using a complex of biotinylated horseradish peroxidase and streptavidin was performed enzymatically according to the manufacturer's instructions and visualized by a color reaction.

RESULTS

A series of preliminary experiments led to the transformation procedures described above. Initial transformation frequencies with pLM2103, a 23-Mdal plasmid coding for lactose utilization, yielded ca. 8.5 transformants per μg of DNA (19). Because of the large plasmid size and the variability in results, we chose pGB301, a 6.5-Mdal *S. sanguis* cloning vector coding for Em^r, to examine the parameters affecting plasmid transformation of *S. lactis* protoplasts.

Plasmid pGB301 was initially isolated from *S. sanguis* Challis SM301 and transformed into *S. lactis* LM0230. Plasmid pGB301 was then isolated from a erythromycin-resistant transformant, *S. lactis* JK301, and restriction endonuclease analysis was performed to confirm uptake of pGB301 and to determine if deletions occurred upon transformation as in the competence-dependent transformation of *S. sanguis* Challis (2) and *Streptococcus pneumoniae* (29). Plasmids pGB301 from *S. sanguis* Challis SM301 and *S. lactis* JK301 were digested with the restriction endonucleases *Hin*dIII and *Bcl*I. Single and double digests were performed and visualized on agarose gels (data not shown). Fragments obtained from both plasmids gave identical electrophoretic patterns and agree with digestion patterns obtained from the known restriction map of pGB301 (3, 4). This result suggests that protoplast transformation in *S. lactis* does not generate deletions.

Plasmid pGB301 was isolated from *S. lactis* JK301 and transformed into *S. lactis* LM0230 at frequencies as high as 1.58×10^3 transformants per μg of DNA. However, in repeated transformation experiments, results were variable. To account for this variability, parameters which affect protoplast regeneration were examined.

Protoplast regeneration studies. The only parameter examined which significantly affected regeneration frequency was the method of plating protoplasts. Table 2 shows a comparison in regeneration frequencies obtained by plating protoplasts with soft agar overlays versus spread plates. Regeneration frequencies were increased from ca. 1% with spread plates to as high as 33% with soft agar overlays.

To simulate transformation conditions, we performed regeneration studies by exposing protoplasts to a 15-min PEG treatment followed by dilution, centrifugation, and suspension as described above for transformation. Table 2 shows that PEG treatment has an adverse effect on regeneration of protoplasts.

Effect of using soft agar overlays versus spread plates on transformation frequency. Since soft agar overlays were shown to enhance the frequency of protoplast regeneration, they were used in transformation experiments to determine if transformation variability could be accounted for by this

TABLE 3. Effect of using soft agar overlays and spread plates on transformation frequency of *S. lactis* LM0230 protoplasts[a]

Expt no.	No. of Em^r transformants	
	Spread plates	Overlays ($\times 10^3$)
1	5.55×10^2	1.27
2	1.10×10^3	3.86
3	1.20×10^3	3.97
4	19	3.24
5	45	1.39
6	<1	2.70

[a] *S. lactis* LM0230 cells were grown for 2 h at 32°C (ca. 2.0×10^8 to 7.0×10^8 CFU/ml), harvested, and treated with mutanolysin to form protoplasts. Conditions of transformation include 750 ng of pGB301 DNA, PEG 3350 at a 22.5% final concentration, and a 2-min PEG treatment time. Transformed protoplasts were incubated for at least 1 h before selection on regeneration plates.

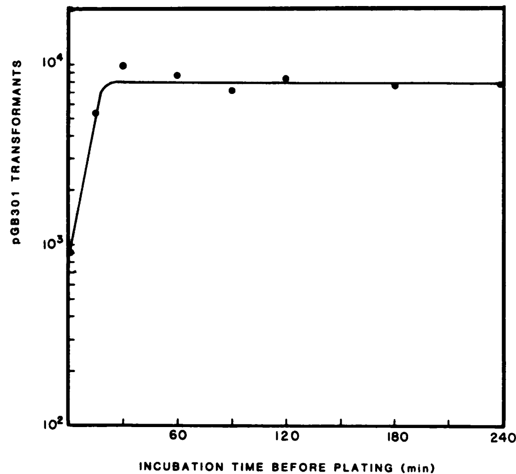

FIG. 1. Time course of appearance of erythromycin resistance phenotype in pGB301 transformants. Protoplasts of *S. lactis* LM0230 were transformed under the conditions described in Table 4, footnote *a*, except 750 ng of pGB301 DNA was used. Transformed cells were allowed to incubate for various time periods in liquid medium before selection on regeneration plates containing erythromycin.

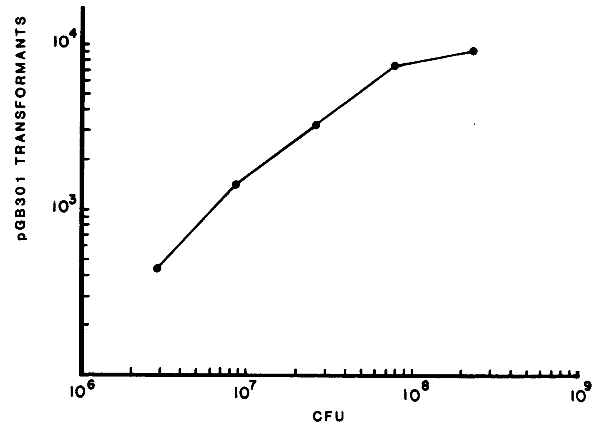

FIG. 3. Effect of *S. lactis* LM0230 protoplast concentration on transformation frequency. Conditions of transformation were as described in Table 4, footnote *a*, except 750 ng of pGB301 DNA was used and protoplast concentrations varied through serial threefold dilutions of a concentrated protoplast suspension. The protoplast concentration (CFU) is the total number of protoplasts used in each 2.0-ml assay.

parameter. Results of six individual experiments are shown in Table 3. Soft agar overlays not only stimulated recovery of transformants but also prevented much of the variability in results obtained with spread plates.

Effect of expression time before plating. The time necessary for phenotypic expression of erythromycin resistance of pGB301 before selection on regeneration plates was determined. After protoplasts were treated with PEG to induce transformation, cells were allowed to incubate for various time periods in antibiotic-free liquid medium before selection on regeneration medium. Results indicate that as incubation time increased before plating, the number of pGB301 transformants increased and reached a maximum after ca. 30 min (Fig. 1). Although less than a 15-min incubation may be adequate for expression of the erythromycin resistance phenotype, we incubated transformed protoplasts for at least 30 min to ensure complete recovery of erythromycin-resistant transformants. The number of transformants recovered did not increase after 4 h, suggesting that *S. lactis* protoplasts do not divide in liquid medium. This was confirmed when no increase in optical density was observed after transformants were selected for in broth and allowed to incubate for extended periods (up to 7 days). The inability of *S. lactis* protoplasts to divide in liquid medium is consistent with results obtained for *Bacillus subtilus* protoplasts (7).

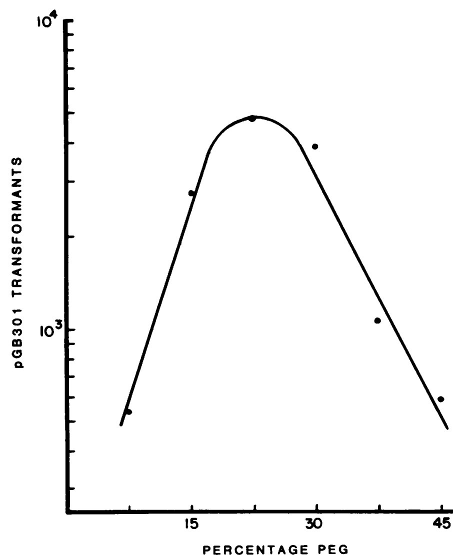

FIG. 2. Effect of PEG concentration on transformation frequency. Protoplasts of *S. lactis* LM0230 were transformed under the conditions described in Table 4, footnote *a*, except 750 ng of pGB301 DNA was used and various concentrations of PEG 3350 were used to induce transformation.

Effect of PEG concentration and molecular weight. PEG molecular weight and concentration were both shown to affect transformation frequency. Initial experiments (with spread plates) showed that PEG, with molecular weights of 3,350 and 6,000 at a 30% final concentration did not significantly affect transformation frequency. PEG with an average molecular weight of 1,000 was less effective, and PEG, with average molecular weights of 300, 600, and 20,000 were ineffective in inducing transformation. In our experiments, we chose PEG with an average molecular weight of 3,350 for use in transformation. Figure 2 indicates that using PEG 3350 at final concentrations of 20 to 30% induced transformation optimally with a peak at 22.5% PEG. Therefore, a final concentration of 22.5% PEG was used in all subsequent experiments.

Effect of protoplast and plasmid DNA concentration. Recently, it was shown that optimal concentrations of protoplasts for transfection of *S. lactis* subsp. *diaetylactis* protoplasts occur at concentrations ranging from 2.0×10^5 to 7.5×10^6 CFU per assay (13). Above these concentrations, a reduction in transfection frequency is observed. To examine this parameter, we performed transformation trials after concentration of protoplasts by centrifugation followed by serial three-fold dilutions to vary protoplast concentration. Results indicate that transformation frequency increases over a range of 3.0×10^6 to 3.0×10^8 protoplasts per assay (Fig. 3).

Quantities of pGB301 DNA ranging from 0.25 to 4.0 μg of DNA per assay were used in transformation of *S. lactis*

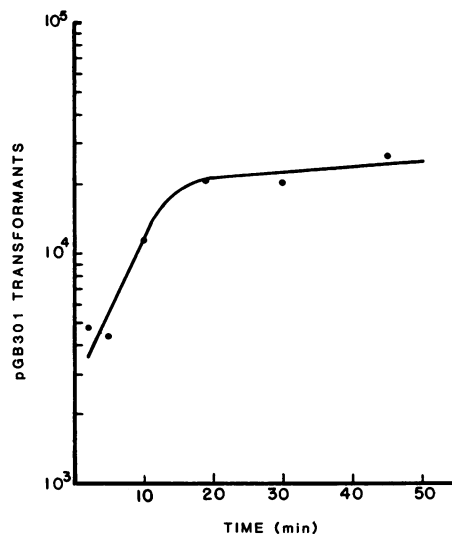

FIG. 4. Effect of PEG treatment time on transformation frequency. Protoplasts of *S. lactis* LM0230 were transformed as described in Table 4, footnote *a*, except 750 ng of pGB301 DNA was used and times for induction of transformation were varied by incubating protoplasts and pGB301 DNA in the presence of PEG for various time periods.

LM0230. Results indicated that transformation frequencies of 3.1×10^2, 8.2×10^2, 1.5×10^3, 4.5×10^3, 9.4×10^3, and 3.5×10^4 were obtained per 0.25, 0.50, 0.75, 1.0, 2.0, and 4.0 μg of DNA, respectively. There appears to be a proportional relationship of transformation frequency to DNA concentration. We commonly used 0.75 to 1.0 μg of DNA to examine transformation parameters.

Effect of PEG-DNA treatment time for induction of transformation. Transformation trials were performed with various exposure times of protoplasts to PEG in the presence of DNA. Figure 4 shows that maximal frequencies of 2.0×10^4 transformants per 750 ng of DNA were achieved only after a minimum PEG-DNA treatment of 20 min. Subsequent experiments were performed with a 20-min PEG-DNA treatment time for maximal transformation.

Transformation of different strains of *S. lactis*. Transformation of five different strains of *S. lactis* was examined. *S. lactis* C20, ML3, and LM0230 were transformed at frequencies of 1.3×10^3, 4.8×10^3, and 3.1×10^4 transformants per 750 ng of DNA, respectively. No transformants were observed for *S. lactis* C10 or M18 with 750 ng of DNA.

TABLE 4. Effect of plasmid size on transformation frequency of *S. lactis* LM0230 protoplasts[a]

Plasmid	Molecular mass (Mdal)	Plasmid-conferred phenotype	No. of transformants per μg of DNA
pPN221	33.0	Lac$^+$	2.2×10^3
pLM2103	23.0	Lac$^+$	4.7×10^3
pAMβ1	17.0	Em^r	1.5×10^4
pGB301	6.5	Em^r	4.1×10^4

[a] *S. lactis* LM0230 was grown to ca. 2.0×10^8 to 7.0×10^8 CFU/ml, harvested, and treated with mutanolysin to form protoplasts. Conditions for transformation include 1.0 μg of plasmid DNA, PEG 3350 at a final concentration of 22.5% and a 20-min PEG treatment time. Transformed protoplasts were incubated for at least 30 min before selection for the Em^r phenotype of pGB301 on regeneration media. Lac$^+$ transformants were selected for on BCP-lactose indicator agar.

TABLE 5. Transformation of *S. lactis* LM0230 with recombinant DNA molecules[a]

Trial	Restriction fragment inserts	No. of transformants	
		Em^r	Lac$^+$
1	*Bcl*I	137	0
	*Bgl*II	75	2
2	*Bcl*I	389	36
	*Bgl*II	778	14

[a] Recombinant DNA molecules were formed by insertion of *Bgl*II or *Bcl*I fragments of pLM2001 into the *Bcl*I site of pGB301. Conditions for transformation were as described in Table 4, footnote *a*. Approximately 1.0 and 3.0 μg of vector DNA were used to form recombinant DNA molecules in trials 1 and 2, respectively. Transformants were obtained by selection for the Em^r marker of the vector followed by replica plating the colonies onto BCP-lactose indicator agar to score for lactose-fermenting ability.

Transformation of linear plasmid DNA. Plasmid pGB301 was cut once with the restriction endonuclease *Bst*EII to linearize the DNA. Generation of linear DNA was confirmed by agarose gel electrophoresis, and transformation of *S. lactis* LM0230 was then performed with both linear and covalently closed circular (CCC) plasmid forms. Results indicated that linear and CCC DNA transformed *S. lactis* LM0230 at frequencies of 2.7×10^2 and 2.8×10^3 transformants per μg of DNA, respectively. This 10-fold-lower transformation of protoplasts by linear plasmid DNA is consistent with results reported for *B. subtilis* (7).

Effect of plasmid size. Four different plasmids of various molecular masses were used in transformation experiments to assess the effect of plasmid molecular mass on transformation frequency. Results in Table 4 show the plasmids used, their molecular mass, their phenotype, and their frequency of transformation. All plasmids transformed *S. lactis* LM0230 at frequencies ranging from 2.2×10^3 to 4.1×10^4 transformants per μg of DNA. Frequencies obtained with the larger-molecular-mass lactose plasmids were the lowest, whereas pGB301, the smallest plasmid used, transformed *S. lactis* at the highest frequency.

Molecular cloning of lactose metabolism. Cloning experiments were designed to insert *Bgl*II and *Bcl*I restriction endonuclease fragments of pLM2001 into the single *Bcl*I site of pGB301. *Bgl*II restriction digestion of pLM2001 yielded five fragments of 17.9, 7.5, 4.5, 1.9, and 1.3 kb (S. K. Harlander et al., submitted for publication), whereas *Bcl*I restriction digestion generated eight fragments of 19.4, 4.4, 2.9, 2.6, 1.3, 0.9, 0.6, and 0.4 kb (S. K. Harlander et al., unpublished data). Two independent cloning trials were performed with both *Bcl*I and *Bgl*II restriction fragments in separate experiments. Recombinant DNA molecules formed were used in transformation of *S. lactis* LM0230 (Table 5). Transformation frequencies were 10- to 100-fold lower than frequencies observed with CCC pGB301.

In the first trial, two Lac$^+$ transformants, designated as JK41 and JK42, were obtained from cloning *Bgl*II fragments of pLM2001. Agarose gel electrophoresis of plasmid DNA isolated from these strains showed that JK41 contained a single plasmid of ca. 24 Mdal (pJK41), whereas JK42 contained a larger plasmid of 35.5 Mdal. Because of the smaller size, pJK41 was analyzed further.

*Bgl*II restriction fragments have compatible ends with *Bcl*I fragments and can be ligated to form a hybrid molecule. However, formation of these hybrid molecules destroys the recognition sequence site for both *Bgl*II and *Bcl*I (23); therefore, a single *Bgl*II fragment which has been inserted into the *Bcl*I site cannot be identified by restriction digestion

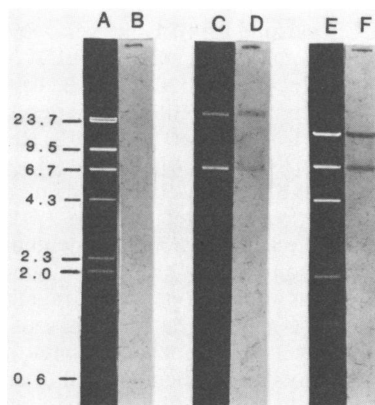

FIG. 5. DNA-DNA blot hybridization of biotinylated pJK41. Agarose gel electrophoretic patterns of: lane A, lambda *Hin*dIII reference markers; lane C, pGB301 nicked circular and CCC plasmid forms; lane E, pLM2001 *Bgl*II restriction fragments of 17.9, 7.5, 4.5, 1.9, and 1.3 kb. After Southern transfer of DNA from a 0.8% agarose gel to nitrocellulose, biotinylated pJK41 was hybridized to: lane B, lambda *Hin*dIII restriction fragments; lane D, pGB301 nicked circular and CCC plasmid forms; lane F, pLM2001 *Bgl*II restriction fragments. Biotinylated pJK41 hybridized to pGB301 vector DNA and the 17.9- and 7.5-kb *Bgl*II fragments of pLM2001. The numbers represent the sizes (kb) and position of reference bands.

with *Bcl*I or *Bgl*II. However, digestion of pJK41 with *Bgl*II yielded two fragments of 7.5 and 28.5 kb. This suggested that three *Bgl*II fragments had been inserted, with the 7.5-kb *Bgl*II fragment of pLM2001 mapping in the center of the other two *Bgl*II fragments inserted.

To verify fragments cloned, a *Bgl*II digestion of pLM2001 was probed with biotinylated pJK41 by Southern blot hybridization (Fig. 5). Probe DNA hybridized to the 17.9- and 7.5-kb *Bgl*II fragments, but a third fragment was not identified. However, agarose gel electrophoresis of a *Bgl*II digestion of pLM2001 with a 2.5% agarose gel revealed that a previously undetected fragment of about 280 base pairs banded between pBR322 *Hin*FI reference fragments F (298 base pairs) and G (221 base pairs). This suggested that this small *Bgl*II fragment could be the third insert fragment (data not shown).

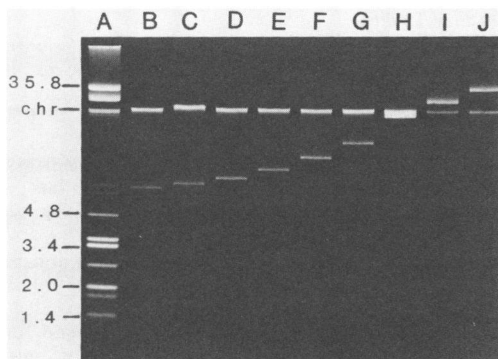

FIG. 6. Agarose gel electrophoretic patterns of various size classes of Lac^- and Lac^+ recombinant plasmids containing *Bgl*II inserts. Lane A, *Escherichia coli* V517 and *E. coli* J53 reference mobility plasmids; lane B, pGB301 vector plasmid; lanes C–H, Lac^- plasmids containing *Bgl*II inserts; lanes I and J, Lac^+ plasmid containing *Bgl*II inserts. Numbers represent the reference plasmid sizes in Mdal. Chr, Chromosome.

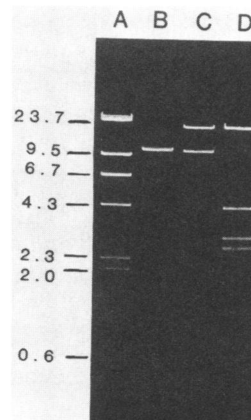

FIG. 7. Agarose gel electrophoretic patterns of *Bcl*I restriction digests of pGB301 (lane B), pJK18 (lane C), and pLM2001 (lane D). *Bcl*I digestion of pJK18 indicates that it is composed of pGB301 and the 19.4-kb *Bcl*I fragment of pLM2001. Bacteriophage lambda digested with *Hin*dIII was used as reference markers (lane A). The numbers represent sizes (kb) and position of marker bands.

Figure 6 shows various size classes of Lac^- and Lac^+ recombinant plasmids containing *Bgl*II fragments which were cloned in LM0230. Plasmid molecular masses ranged from 6.6 to 37.5 Mdal. Various size classes of Lac^- plasmids containing *Bcl*I inserts were also observed (unpublished data).

In the second trial, 36 Lac^+ transformants were obtained which contained plasmids with *Bgl*II inserts. These plasmids ranged in molecular mass from 20.5 to 30 Mdal. Further analysis of fragments cloned was not pursued since plasmid sizes did not suggest that single *Bgl*II inserts were obtained. Lac^+ transformants with plasmids containing *Bcl*I inserts ranged in molecular mass from 18.5 to 26.5 Mdal. A plasmid of 18.5 Mdal, pJK18, was digested with *Bcl*I to determine fragments cloned. Results show that pJK18 had a single 19.4-kb *Bcl*I insert (Fig. 7). These results indicate that pGB301 is a suitable vector for cloning in *S. lactis* and that the transformation frequency is sufficient for cloning plasmid-coded genes.

DISCUSSION

Initial studies in our laboratory showed that plasmid transformation procedures effective with other groups of microorganisms are unsatisfactory for transformation in *S. lactis* (unpublished data). Plasmid transformation of protoplasts appeared to be an attractive approach since it does not require cell competence for DNA uptake (15). Shortly after reporting on conditions for efficient protoplast formation (20), we reported on the PEG-induced transformation of protoplasts with plasmid DNA coding for lactose metabolism (19). However, transformation frequencies were low and quite variable in subsequent experiments. In this study, we examined factors affecting the regeneration of protoplasts on solid media and used pGB301, a 6.5-Mdal *S. sanguis* cloning vector which is able to replicate and express Em^r in *S. lactis*, to optimize several parameters which affect transformation frequency. Use of soft agar overlays enhanced regeneration of protoplasts and was significant in obtaining consistent reproducible transformation results.

PEG treatment is essential for transformation of *S. lactis* protoplasts (19). The mechanism by which PEG induces transformation is still unknown, but it may cause the cell membrane to become more permeable to DNA, or alterna-

tively, it may cause a conformational change in the DNA molecule which allows penetration into the protoplast (5). In this study, PEG concentration, molecular weight, and treatment time were all shown to be critical for increasing transformation frequency. The optimal concentration of PEG was found to be 22.5% when using PEG with an average molecular weight of 3,350. This is within the range observed for optimal PEG-induced transformation in *Streptomyces* spp. (20% PEG) and *B. subtilis* (30% PEG) (15). Higher concentrations of PEG have been shown to enhance protoplast fusions, therefore suggesting that PEG-induced protoplast fusion and transformation occur by different mechanisms (15). However, a 36% final PEG concentration was shown to be optimal for transfection of *Acholeplasma laidlawii* (30).

The time of PEG-DNA treatment of protoplasts was shown to affect transformation frequency significantly. A 1- to 2-min PEG-DNA treatment is commonly used to induce transformation in other genera (5–7, 14–16, 27), a 45- to 60-s PEG-DNA treatment has been shown to be optimal for transfection in *Streptomyces* spp. (32), and a 10-min PEG-DNA treatment is used in transfection of *S. lactis* subsp. *diacetylactis* (13). Our results indicate that a 1- to 2-min PEG-DNA treatment time is insufficient for maximal transformation in *S. lactis*. Although PEG had an adverse effect on regeneration frequency, maximal transformation frequencies were achieved only after a minimum treatment time of 20 min. It is possible that membrane composition in *S. lactis* may be sufficiently different to cause the increased time for PEG induction of transformation.

Attempted transformation of different strains of *S. lactis* revealed that not all strains could be transformed by pGB301. This suggests that pGB301 may be restricted upon entry, that pGB301 is incompatible with resident plasmids in the nontransformable strains, or that the transformation efficiency is low at the DNA concentrations used. The strains which could be transformed (LM0230, ML3, and C20) have been shown to exchange genetic information through conjugation (unpublished data) and have been suggested to be genetically related (9). It is also possible that only certain strains are capable of being transformed or capable of regenerating cell walls with the protocol employed.

Although we have been able to increase the frequency of plasmid transformation of *S. lactis* protoplasts to as high as 4.1×10^4 transformants per μg of DNA, the frequency of transformation differs markedly from those obtained for *Streptomyces* spp. (5), *Bacillus stearothermophilus* (16), and *B. subtilis* (7) in which much higher frequency of protoplast transformation is observed. However, protoplast transformation frequencies in *Staphylococcus carnosus* (14) and other *Bacillus* species (6, 27) and transfection frequencies in *S. lactis* subsp. *diacetylactis* (13), *Streptomyces* spp. (32), and *A. laidlawii* (30) are very similar. The reason why some species can be transformed at high frequencies and other species are transformed at frequencies several orders of magnitude lower is unknown. Suarez and Chater (32) suggested that a subpopulation of *Streptomyces* protoplasts are competent for PEG-induced transfection; therefore, it may be possible that a similar phenomenon occurs in *S. lactis* and other species. Further research is needed to determine the mechanism of PEG-induced transformation. This could provide explanations for the observed differences in transformation frequencies.

The frequencies of transformation with linear and recombinant plasmid DNA appear to be 10- to 100-fold lower than that with the CCC plasmid form. However, we were able to clone plasmid-coded lactose genes from pLM2001 with the *S. sanguis* cloning vector, pGB301. The lactose-metabolizing genes were tentatively located on a 7.5- or a 17.9-kb *Bgl*II and a 19.4-kb *Bcl*I fragment of pLM2001. Recent results from our laboratory (S. K. Harlander et al., unpublished data) indicate that the 19.4-kb *Bcl*I fragment overlaps the entire 17.9-kb *Bgl*II fragment but not the 7.5-kb fragment. This suggests that the lactose-metabolizing genes reside on the 17.9-kb but not on the 7.5-kb *Bgl*II fragment. Experiments are in progress to subclone these fragments to more precisely locate these genes. The frequency of transformation is now sufficient to clone plasmid-linked genes. Since many important properties associated with dairy fermentations are plasmid mediated (24), the ability to clone plasmid-coded genes in *S. lactis* is essential for eventual improvement of strains used in dairy fermentation processes.

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