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A Method for Genetic Transformation of Nonprotoplasted *Streptococcus lactis*

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Plasmid transformation of whole cells of *Streptococcus lactis* LM0230 was demonstrated. The procedure required polyethylene glycol and incubation in hypertonic media, but did not require enzymatic cell wall digestion. Conditions were optimized, yielding 5×10^5 transformants per μg of pSA3 DNA. Variables tested for effect on transformation efficiency included molecular weight, concentration, and pH of polyethylene glycol; cell density; plating media; DNA concentration; heat shock; and incubation of cells in hypertonic buffer. DNAs transformed included pSA3, pVA856, pTV1, and c2ϕ. Transformation from DNA-DNA ligation mixes, with DNA not purified through density gradients, and with previously frozen cells was also achieved. The method described here for transformation of nonprotoplasted cells of LM0230 is unique, and to date has not been applied successfully to other lactic acid bacteria.

The identification of a plasmid DNA transformation system for the group N streptococci is essential for the application of in vitro recombinant DNA technology for the improvement of these bacteria. Early investigations into the transformation of *Streptococcus lactis* suggested that whole-cell procedures were unsatisfactory (15). Similar findings for *Lactobacillus casei* also were inferred (16). Owing to the negative nature of these results, methodologies and data for nonprotoplast transformation systems for the lactic acid bacteria were not presented. Since that time it has generally been assumed that transformation of the lactic acid bacteria is limited to protoplast-dependent procedures, justifying numerous studies on protoplast formation and regeneration for a variety of lactic acid bacteria strains (7, 14, 16, 21). This approach led to the unequivocal establishment of protoplast transformation of *S. lactis*, first by Kondo and McKay (15), followed by reports from several other laboratories (3, 9, 12, 25, 31).

The maximum efficiency of protoplast transformation in *S. lactis* was reported by Simon et al. (25), who were able to obtain 5×10^6 erythromycin-resistant (Em^r) transformants per μg of pIL204 DNA. Transformation frequencies were included in only two other reports, that of Yu et al. (30), who recovered approximately eight lactose-positive (Lac^+) transformants per μg of DNA, and that of Kondo and McKay (13), who recovered 4×10^4 transformants per μg of pGB301 DNA (2).

Although transformation of *S. lactis* with plasmid DNA has been accomplished, the poor understanding of protoplast formation and regeneration conditions, as well as DNA uptake mechanisms, has in practice caused difficulties in reproducing established protocols in different laboratories (30; M. E. Sanders and M. A. Nicholson, unpublished observations). In an effort to modify published transformation systems for effectiveness in our hands, we identified a plasmid transformation system which does not require protoplast formation of *S. lactis* cells. Since protoplast formation and regeneration are not components of the transformation system, simplicity and reproducibility were superior to those of protoplast-dependent protocols. Transformation frequencies of 5×10^5 transformants per μg of DNA were obtained for intact, cesium chloride gradient-purified pSA3 plasmid DNA into *S. lactis* LM0230. This paper describes the first report of a whole-cell plasmid transformation system for lactic acid bacteria.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *S. lactis* LM0230 was obtained from L. L. McKay, University of Minnesota. The strain was propagated at 32°C in M17 broth (Difco Laboratories, Detroit, Mich.) with 0.5% glucose (M17-glc). All transformations were conducted with LM0230. Plasmids used for transformations are listed in Table 1. Optical densities were recorded on a Spectronic 20 spectrophotometer (Busch & Lomb, Inc., Rochester, N.Y.).

DNA isolation. pSA3, pAN50, and pVA856 plasmid DNAs were isolated from *Escherichia coli* strains by the potassium acetate procedure outlined by Silhavy et al. (24). Small-scale DNA isolations from 5 ml of culture were prepared for quick plasmid screening. For DNA used in transformations, the protocol was scaled up for 1 liter of cells. Isopropanol-precipitated DNA was loaded into a 40-ml cesium chloride-ethidium bromide gradient (refractive index, 1.3970) and centrifuged in a Beckman Ti60 rotor at 47,000 rpm for 48 h. DNA was dialyzed overnight in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and precipitated with 2 volumes of 95% ethanol and 0.1 volume of 5 M ammonium acetate. DNA was suspended in TE buffer to the indicated concentrations. Chromosomal DNA was isolated from LM0230 transformants by a modification of the Marmur (19) method. A 10% inoculum from overnight M17-glc plus antibiotic culture was made into 9 ml of M17-glc broth plus 20 mM DL-threonine, and cultures were incubated for 2 h at 32°C. Cells were harvested, suspended in 1 ml of cold 0.01 M Tris (pH 7.0), and transferred to 1.5-ml tubes. Cells were pelleted and suspended in 500 μl of 25% sucrose–5.0 mM Tris (pH 8.0)–2 mM EDTA. To these cells, 6 μl of RNase A (10 mg/ml; Sigma Chemical Co., St. Louis, Mo.) and 70 μl of lysozyme (15 mg/ml in water; Sigma) were added, and the suspension was held for 1 h at 37°C. Then 4 μl of 10% sodium dodecyl sulfate was added. The suspension was inverted to mix it and held at room temperature for 20 min. DNA was fragmented by vortexing at maximum speed for 20 s. Two extractions with 50 mM Tris (pH 8.0)–saturated phenol-chloroform (1:1,

TABLE 1. Plasmid descriptions

Plasmid (kb[a])	Markers[b]	Reference
pSA3 (10.2)	Em^r	5
pVA856 (9.1)	Em^r	17
pAN50 (13.2)	Em^r Cm^r	Sanders and Nicholson, unpublished data
pTV1 (12.4)	Em^r Cm^r	29
pME1a (16.0)	Phage resistance	22

[a] kb, Kilobases.
[b] Expressed in streptococci. Cm^r, Chloramphenicol resistance; Em^r, erythromycin resistance.

vol/vol) were followed by three chloroform-isoamyl alcohol (24:1, vol/vol) extractions. DNA was passed through a Centricon filter (Amicon Corp., Danvers, Mass.) and concentrated to 100 μl. pME1a was purified from LM0230 by the lysis and CsCl banding procedures of Klaenhammer et al. (10). Plasmid DNA was isolated from other *S. lactis* LM0230 derivatives by the procedure of Anderson and McKay (1), with the following modifications to render the DNA amenable to digest with many restriction enzymes. A 10% inoculum of overnight cells was made into 10 ml of M17-glc broth. Cells were grown for 2 h at 32°C, pelleted, and suspended in 379 μl of 6.7% sucrose (in 50 mM Tris–1 mM EDTA, pH 8.0). Lysozyme (10 mg/ml in 25 mM Tris, pH 8.0; Sigma) was added (96.5 μl), and cells were incubated for 30 min at 37°C. EDTA (0.25 M in 50 mM Tris, pH 8.0) was added (48.2 μl), followed by 27.6 μl of 20% sodium dodecyl sulfate (wt/vol in 50 mM Tris–10 mM EDTA, pH 8.0). NaOH (3.0 N) was added (27.6 ml), and the tubes were slowly inverted for 10 min. A 49.6-μl portion of a 2.0 M Tris (pH 7.0) solution was added, and the tubes were mixed by gentle inversion for 3 min. A 71.7-μl portion of 5.0 M NaCl was added, and the tubes were placed on ice for 30 min. A cleared lysate was obtained after centrifugation for 20 min in an Eppendorf 5412 centrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.). The aqueous phase was extracted sequentially with phenol (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) saturated with 3% NaCl and chloroform-isoamyl alcohol (24:1, vol/vol), and the DNA was precipitated with 2 volumes of 95% ethanol and 0.05 volume of 5 M ammonium acetate. Samples were placed at −20°C for 1 h and were centrifuged for 20 min to pellet DNA. The pellet was washed once in cold 70% ethanol, dried under vacuum, and suspended in 20 to 30 μl of TE buffer containing 0.5 mg of RNase A per ml prepared as described previously (18). Restriction enzyme digestion of 5 μg of plasmid DNA prepared in this manner yielded completely digested visible bands on agarose gels.

To prepare DNA from bacteriophage c2ϕ, we lysed a 10-ml M17-glc broth culture of LM0230 with c2ϕ. After cell clearing, debris was removed by filtration of the lysate through a 0.45-μm-pore-size filter. The phage lysate was aliquoted at 1 ml per 1.5-ml tube. To the lysate were added 30 μl of RNase A (1 mg/ml) and 200 μl of polyethylene glycol (PEG)-NaCl solution (20% PEG [molecular weight, 8,000], 2.5 M NaCl, passed through a 0.45-μm-pore-size filter). The mixture was inverted several times to mix, incubated for 15 min at room temperature, and centrifuged in an Epperdorf 5412 centrifuge for 5 min. The supernatant fluid was removed with an aspirator. Protease type XI (Sigma; equivalent to proteinase K) was added (200 μl of a 50-μg/ml solution in 10 mM Tris–1 mM EDTA–0.2% Sarkosyl, pH 7.8) and mixed with vigorous vortexing. This mixture was heated at 55°C for 30 min, and 26 μl of 3 M NaCl was added. Phenol saturated with TE buffer was added (100 μl) and mixed in vigorously. Phage were allowed to incubate on phenol for 15 min. Chloroform (100 μl) was added, the mixture was centrifuged for 5 min, and the aqueous layer was removed and extracted once with chloroform and once with ether. DNA was precipitated with 2 volumes of 95% ethanol at −20°C for 1 h. The pellet was washed with 1 ml of 70% ethanol, dried, and suspended in 40 μl of TE buffer.

DNA concentrations were measured at 525 nm on a spectrophotofluorometer (Aminco-Bowman, Silver Spring, Md.) in 5 mM Tris–0.5 mM EDTA–0.5 μg of ethidium bromide per ml (pH 8.0) against DNA standards.

Transformation. The optimized transformation protocol for pSA3 DNA into LM0230 cells was conducted as follows. Buffer systems were adapted from procedures described by Kondo and McKay (15) and Wirth et al. (28). LM0230 was inoculated from a frozen seed stock into M17-glc broth and grown overnight at 32°C. A 0.3-ml sample was transferred into 30 ml of M17-glc broth and grown at 32°C for 4 h (optical density at 600 nm, 0.9). Cells were harvested by centrifugation at 4,340 × *g*. Cell pellets were suspended in 7.6 ml of 0.5 M sucrose (in 0.01 M Tris, pH 7.0) and incubated for 60 min at 37°C. Cells were centrifuged at 3,020 × *g* for 10 min and suspended in 1 ml of SMMB (0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$ [pH 6.5] with 5% bovine serum albumin). Cell suspensions were divided into 0.5-ml aliquots into clean, sterile tubes, and DNA (0.5 to 4 μg) mixed at equal volume with double-strength (2×) SMM (1.0 M sucrose, 0.04 M maleate, 0.04 M $MgCl_2$, pH 6.5) was added. To this mixture, 1.8 ml of 30% PEG with an average molecular weight of 8,000 (PEG 8000) in TSCM (100 mM Tris, 40 mM $CaCl_2$, 10 mM $MgCl_2$, 500 mM sodium succinate, pH 6.5) was added. The cell-DNA-PEG mixture was incubated at room temperature for 20 min and then heat shocked (5 min at 42°C). A 5-ml portion of SMMB was added, and the suspension was mixed and centrifuged at 3,020 × *g* for 10 min. Cells were washed with 5 ml of SMMB and centrifuged at 3,020 × *g* for 10 min. Pellets were resuspended in 1 ml of an equal mixture of 2× SMM and M17-glc broth. Erythromycin was added to a final concentration of 0.05 μg/ml, and cells were incubated for 30 min at room temperature for expression. Cells were plated by adding 12 ml of SM17-glc (M17-glc supplemented with 0.5 M sucrose) overlay agar (0.5% agar) directly to the tube and plating 3 ml per plate on SM17-glc bottom agar (1.5% agar). Alternatively, the transformation mix was serially diluted in SMMB, mixed with 3 ml of overlay, and plated on bottom agar. Plates were incubated for 3 to 4 days at 32°C.

Centrifugation steps were conducted at 15°C. All solutions except PEG and 0.01 M Tris were stored at 4°C and allowed to warm to room temperature before use. The PEG solution was aliquoted into 2-ml quantities, stored at −20°C, thawed on ice, and used cold. The 0.01 M Tris solution used for washing cells was stored at 4°C until used. Tris-sucrose, SMM, SMMB, and PEG solutions were filtered through 0.45-μm-pore-size filters. TSCM buffer was prepared by autoclaving succinate and other salts separately, tempering at 45°C, and combining. All reagents were purchased from Sigma except PEG which was purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.). All experiments included controls in which DNA was omitted. Em^r mutants never occurred. All reagents were plated during each experiment to verify reagent sterility. Variations from this protocol are indicated when applicable.

DNA manipulations. A nick translation kit from Amersham Corp. (Arlington Heights, Ill.) was used to label pTV1 DNA

TABLE 2. Dependence on enzyme treatment of c2ϕ DNA entry into LM0230

Enzyme used	PFU/ml			
	+Enzyme		−Enzyme	
	+DNA	−DNA	+DNA	−DNA
Mutanolysin[a]	300	<10	<10	<10
Novozyme[b]	70	<10	350	<10

[a] Protocol used was according to Kondo and McKay (13) with the following modifications. LM0230 indicator cells were plated with transfectants on M17-glc for plaque detection; mutanolysin was used at a final concentration of 6.25 μg/ml. No-enzyme control was incubated at 37°C for 20 min in SMMB without mutanolysin.

[b] Protocol used was as described in Materials and Methods with the following modifications. Novozyme (0.66 mg/ml, final concentration) was added before 1 h of incubation at 37°C; heat shock was omitted; 35% PEG 1000 at pH 7.5 was used instead of 30% PEG 8000 at pH 6.5; indicator cells were plated with transfectants on M17-glc agar. The no-enzyme control was incubated at 37°C for 60 min in SMMB without Novozyme.

with [α^{32}P]dCTP. DNA-DNA hybridizations were conducted as previously described (23). Chromosomal DNA was digested with *Eco*RI (New England BioLabs, Inc., Beverly, Mass.) according to the specifications of the supplier. Cloning reactions were done by the method of Maniatis et al. (18), using T4 DNA ligase (New England BioLabs).

RESULTS

Establishment of whole-cell transformation. In initial studies, the Kondo and McKay (13) procedure was modified with respect to incubation time with enzyme, use of Novozyme 234 (Novo Laboratories, Inc., Wilton, Conn.) instead of mutanolysin, and PEG. The procedure was applied to a phage c2ϕ transfection system to eliminate the variable of regeneration efficiency. To control for enzyme effects, samples omitting enzyme were included. The results (Table 2) showed that the procedure of Kondo and McKay (13) with mutanolysin (6.25 μg/ml) yielded a higher level of transfectants than the modified procedure with Novozyme 234. However, omitting the Novozyme 234 from the modified procedure resulted in the highest levels of transfectants recovered. Omitting mutanolysin from the procedure of Kondo and McKay (13) failed to produce transfectants, suggesting that the modified procedure rendered whole cells of *S. lactis* LM0230 transformable. In comparing the modified transformation procedure with that reported by Kondo and McKay (13), the differences appeared minor, yet no transfectants were detected when the Kondo and McKay procedure was used without enzyme treatment. Differences in the protocols included the following. (i) Cells were washed after growth with cold 0.01 M Tris, not cold water; (ii) 1.8 ml of 35% PEG 1000 in a succinate-based buffer (TSCM; see Materials and Methods) held at 0°C was used, instead of 30% PEG 3350 in a sucrose-based buffer at room temperature; and (iii) cells were incubated in sucrose buffer for 1 h.

TABLE 3. Transfection[a] of LM0230 with c2ϕ DNA and 30 and 35% PEG at room temperature (RT) and 0°C

% PEG (temp)	With DNA		Without DNA
	Total	Per μg[b]	(Total)
30			
RT	320	52	ND[c]
0°C	1,000	100	<10
35			
RT	880	140	ND
0°C	1,600	260	<10
None	<10	<0.16	<10

[a] Procedure used as described in Materials and Methods with the following modifications. 35% PEG was made of PEG 1000 in TSCM (pH 7.5); 30% PEG was made of PEG 3350 in SMM, autoclaved, and stored and used at room temperature as per Kondo and McKay (13); no heat shock was incorporated.

[b] 6.2 μg of DNA used per experiment.

[c] ND, Not determined.

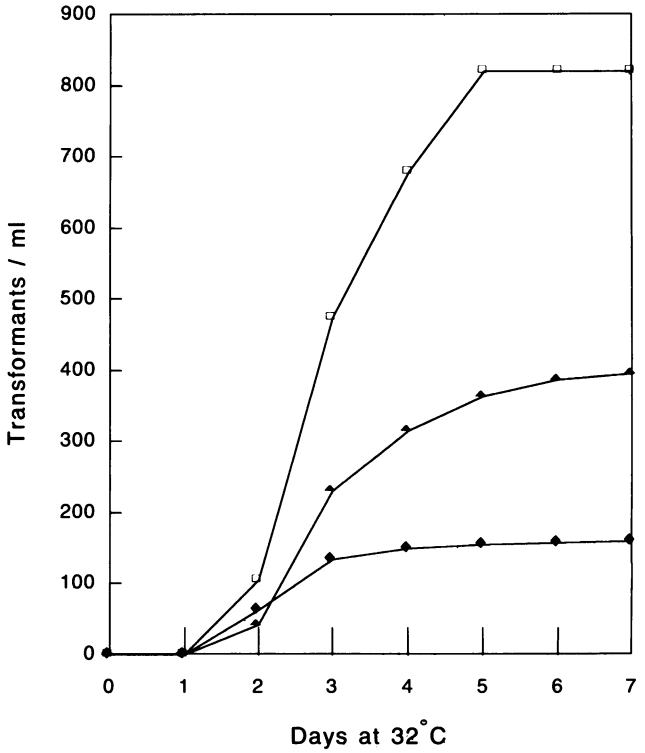

FIG. 1. Dependence of whole-cell transformation on heat shock, time of incubation, and recovery medium. Transformation was conducted as indicated in Materials and Methods except that cells were grown for 2 h before harvesting and 35% PEG 1000 (pH 7.5) was used. Symbols: □, transformants underwent heat shock; ▲, ◆, no heat shock used; □, ▲, transformants plated on SM17-glc; ◆, transformants plated on M17-glc.

To ascertain the importance of PEG differences in whole-cell transfection, we compared 30% PEG prepared as per Kondo and McKay (13) and 35% PEG prepared as in Materials and Methods. The results (Table 3) show that whole cells took up c2ϕ DNA with both PEG solutions at both room temperature and 0°C, but not in the absence of PEG. The highest efficiency was seen with 35% PEG at 0°C. The results suggested that differences other than PEG differences between the protocol presented here and that described by Kondo and McKay (13) were sufficient to account for successful whole-cell transfection.

Optimization of transformation. Subsequent experiments were designed to demonstrate utility of the whole-cell protocol with plasmid DNA uptake and to optimize transformation efficiency. The effects of plating media, time of incubation, and heat shock on transformation efficiency were tested. Transformants were recovered at significantly higher levels when plated on a medium with high osmotic stability (SM17) versus standard plating medium (M17) (Fig. 1). The medium described by Okamoto et al. (21) did not show

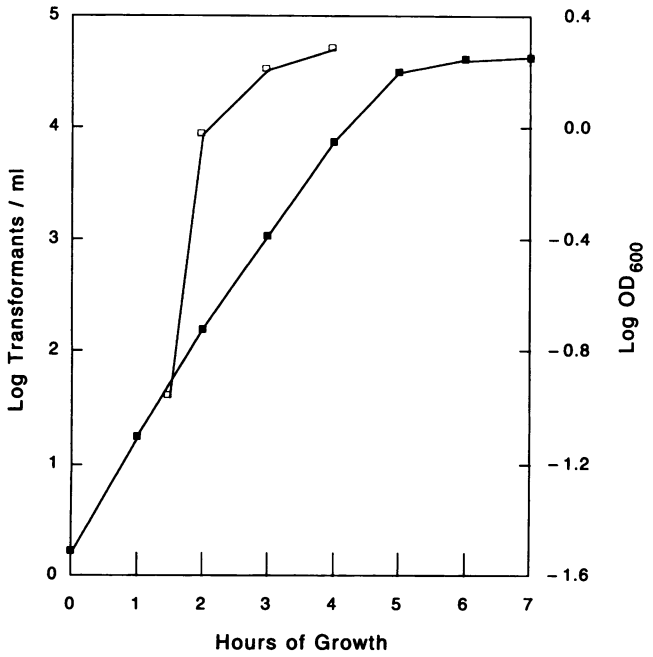

FIG. 2. Effect of cell density on transformation. Transformation was conducted as described in Materials and Methods. Symbols: ■, cell density (log optical density at 600 nm [OD_{600}]); □, log transformants per ml.

greater transformant recovery in a parallel experiment (data not shown) and therefore was not implemented on a regular basis. Levels of transformants steadily increased up to 5 days at 32°C. However, depending on desired yields, plates could be examined after only 3 days. Colony development was slower at 28°C (data not shown), although a previous report suggested an advantage of 28°C over 32°C for recovery of regenerated protoplast transformants (13). Finally, Fig. 1 shows a positive effect of a heat shock treatment (42°C for 5 min) on transformant recovery. Levels of transformants more than doubled with this treatment. Therefore, this step was included in subsequent experiments.

The effect of PEG concentration was further investigated. Of 0, 10, 20, 30, and 35% PEG 8000 solutions prepared in TSCM, 30% PEG 8000 gave maximum transformant yields. No transformants were recovered when 0% PEG was used. PEG was effective over a wide range of pH values (5.5 to 8.3). However, a pH of 4.4 completely prevented transformation. The dependency of transformation on the molecular weight of PEG was also tested. Solutions (35%; pH 7.5) of PEG with average molecular weights of 1,000, 3,350, and 8,000 were tested. Cells were grown for 2 h before the experiment, and 2.2 μg of pSA3 DNA was used per experiment. With PEG 1000, 4.9×10^2 transformants per ml were obtained; with PEG 3350, 1.5×10^3 transformants per ml were obtained. Maximal yields of transformants were obtained with PEG 8000 (2.4×10^3 transformants per ml). Furthermore, PEG 3350 made in TSCM with sucrose instead of succinate yielded 10-fold fewer transformants (2.6×10^3/ml). Less than one transformant per ml was seen when no DNA was added. Therefore, the succinate appears to aid in DNA uptake.

To determine the effect of cell density on transformation efficiency, we grew LM0230 cells at 1% inoculum for 1.5, 2.0, 3.0, and 4.0 h at 32°C. Transformants were recovered at greater efficiencies at the highest cell densities tested (Fig. 2). The 4.0-h-old cells corresponded to an A_{600} of 0.88, with 10^8 viable cells per ml. This contrasts with protoplast procedures in which low cell densities were recommended (13).

TABLE 4. Use of whole-cell transformation of LM0230 for direct cloning of pME1a onto pSA3

Cloning reaction	Amt pSA3 used (μg)	No. of transformants/μg	No. of transformants/ml
pSA3 (CsCl)	0.5	5.2×10^5	2.6×10^5
pSA3 (religated)	2.4	3.3×10^3	7.8×10^3
pSA3 + pME1[a]	0.6	5.8×10^3	3.5×10^3

[a] 12.4 μg of pME1a was ligated to pSA3.

In an attempt to shorten the protocol, the requirement for cell incubation in 0.5 M sucrose before DNA addition was tested. It was found that shortening the holding time in sucrose at 37°C from 60 to 15 min resulted in almost a 2 log cycle drop in transformant recovery (8.0×10^4 to 9.5×10^2 transformants per ml, respectively). Therefore, this step appeared to be important in cell adaptation to transformability.

The ability to use frozen cells of LM0230 as recipients of transformation was tested. Cells grown to normal levels were pelleted and frozen overnight at −70°C as pellets (no stabilizer) or in 0.5 M sucrose–0.01 M Tris (pH 7.0). Cells were thawed and transformed with 0.5 μg of pSA3 DNA along with freshly grown cells as a control. Both frozen cell preparations transformed equivalently well, at levels approximately 10-fold lower than control counts. The transformability of pSA3 isolated from 10 ml of LM0230 cells by the procedure of Anderson and McKay (1) without purification of the plasmid through a CsCl-ethidium bromide gradient was determined. A total of 7.1×10^3 transformants per ml were obtained from plasmid DNA from 10 ml of cells. Therefore, purification of DNA through density gradients is not required for transformation, although transformation efficiency is decreased.

Applicability of transformation system to cloning strategies. The ability to apply this transformation system to nonsupercoiled DNA from ligation reaction mixes would enable direct cloning of genes into LM0230 without prior selection in *E. coli*. To test whether this transformation system was suitable for DNA in this form, we mixed pSA3 (0.6 μg; linearized with *Eco*RV) with pME1a (12.4 μg; digested with *Eco*RV). This DNA mixture was incubated overnight with 400 U of T4 DNA ligase. As controls, CsCl-purified pSA3 (0.5 μg) and pSA3 religated to itself (2.4 μg) were tested. DNA was transformed by the standard protocol into LM0230 with selection for Em^r. The results (Table 4) show that linearized DNA transformed at approximately a 2 log cycle lower efficiency. However, the levels of transformants obtained were suitable for establishing a partial gene bank of pME1a into pSA3. Twenty-one transformants selected at random were assayed for plasmid content. Plasmid DNA was prepared by modifications of the procedure of Anderson and McKay (1) described in Materials and Methods. This DNA was digested with *Eco*RV. Of those transformants tested, six showed insert DNA (four unique clones shown in Fig. 3). This evidence establishes the utility of this transformation system for direct cloning into *S. lactis*.

It was of interest to determine whether this DNA uptake system was applicable to DNAs other than c2ϕ DNA and pSA3. Therefore, additional plasmids were tested for transformability into LM0230, including pVA856, pAN50,

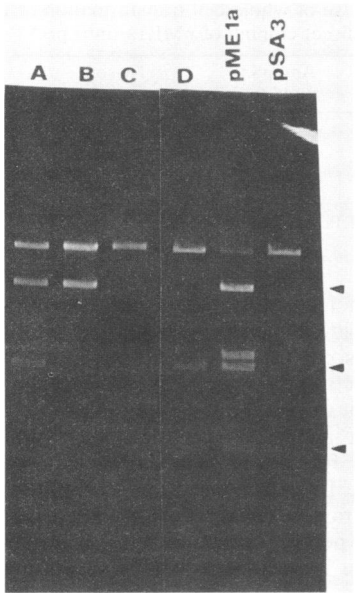

FIG. 3. Agarose gel electrophoresis of *Eco*RV-digested plasmid DNA from pSA3, pME1a, and pSA3 containing different fragments of pME1a (lanes A, B, C, and D). Arrowheads show positions of cloned fragments.

and pTV1. Although the frequencies of transformation of these plasmids were lower, in each case transformants were isolated. The presence of plasmid DNA was confirmed by agarose gel electrophoresis (Fig. 4) for pSA3, pVA856, and pAN50 transformants. Restriction digests of transformed plasmids were indistinguishable from original input DNA (data not shown), suggesting that DNA rearrangements do not occur with this transformation method.

When Em^r transformants of pTV1, a Tn*917* delivery vehicle, were tested for plasmid DNA, none was apparent.

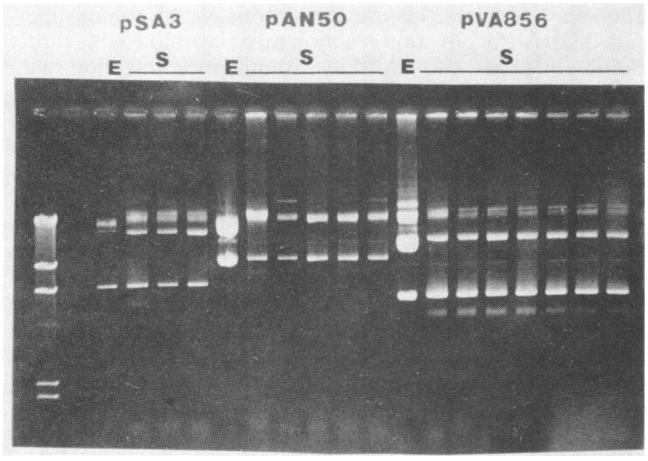

FIG. 4. Agarose gel electrophoresis of plasmid DNA isolated from pSA3, pAN50, and PVA856 transformants of LM0230. (E) Lanes contain cesium chloride gradient-purified plasmid DNA from the *E. coli* source of the plasmid. This DNA was used in the transformation reactions. (S) Lanes contain plasmid DNA from respective *S. lactis* LM0230 transformants. The first lane contains λ DNA digested by *Hin*dIII as mobility standard. Plasmid DNA from LM0230 transformants was isolated by the method of Anderson and McKay (1).

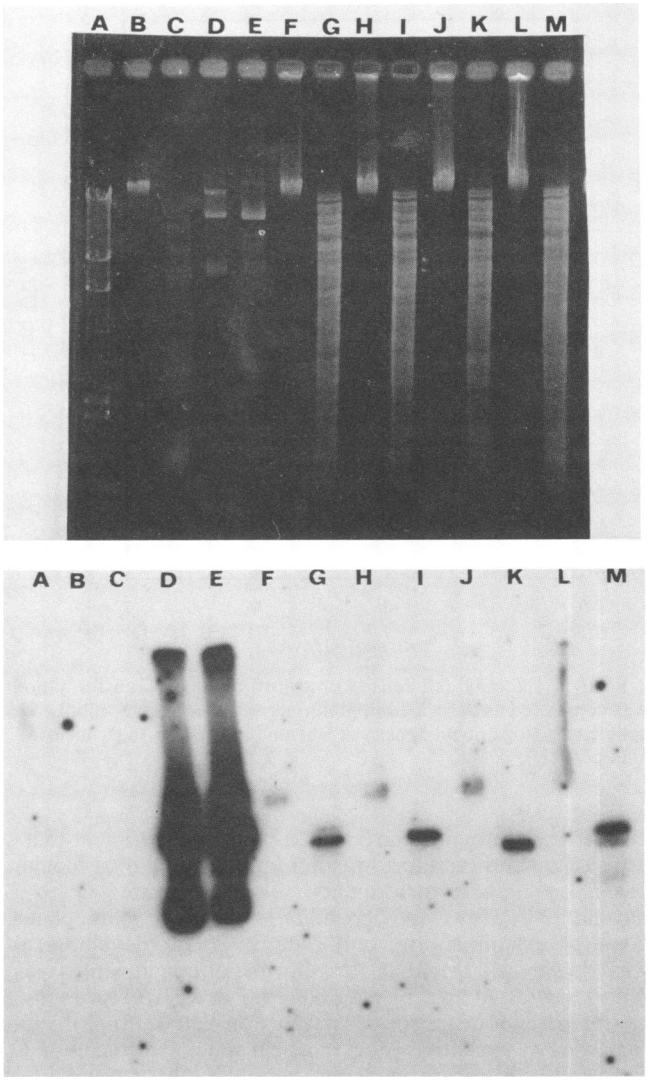

FIG. 5. Agarose gel electrophoresis (top) and autoradiography (bottom) of chromosomal DNA from PTV1 transformants. Chromosomal DNA was prepared from four Em^r transformants of pTV1 (lanes F through M). Lanes D and E contain pTV1 DNA. Lanes F, H, J, and L contain chromosomal DNA from independent transformants. Lanes G, I, K, and M contain *Eco*RI-digested chromosomal DNA from strains shown in lanes F, H, J, and L, respectively. Chromosomal DNA from LM0230 is shown in lanes B (uncut) and C (cut with *Eco*RI). The ethidium bromide-stained gel (top) was dried and probed with nick-translated pTV1. Lane A contains λ DNA digested with *Hin*dIII.

Transformants were selected based on erythromycin resistance, a marker maintained on Tn*917*. Therefore, if transformation was followed by transposition, the Em^r phenotype could result from Tn*917* carried on the chromosome. Chromosomal DNA from four of these transformants was prepared, cut with *Eco*RI, and probed with ^{32}P- labeled pTV1. The results (Fig. 5) show that pTV1 DNA is present in chromosomal DNA preparations of the transformants, since the hybridizing EcoRI fragments are of different sizes from each other and from pTV1. Whether the integration of DNA from pTV1 was due to transposition or homologous recombination is not known. However, the recovery of a single hybridizing band reinforces the view that Tn*917* transposed

and that intact pTV1 is not integrating. Since pTV1 has a single *Eco*RI site near but outside Tn*917*, two junction fragments would probably result from homologous integration.

DISCUSSION

This study constitutes the first report, to our knowledge, of a nonprotoplast transformation procedure for the lactic acid bacteria. PEG-dependent, nonprotoplast transformation procedures have been described for such diverse microbes as *Bacillus brevis* (27), *E. coli* (11), *Clostridium thermohydrosulfuricum* (26), *Saccharomyces cerevisiae* (11), *Rhodopseudomonas sphaeroides* (6), and *Kluyveromyces lactis* (8). The specifics of these protocols vary, but in several cases, preconditioning of the cells in certain buffers was essential for transformation (6, 26, 27). Also, each procedure exhibited an absolute requirement for PEG treatment, distinguishing this group of procedures from whole-cell procedures which require treatment with alkaline cations, but not with PEG, and from protoplast procedures which require cell wall digestion. For the procedure described here for *S. lactis*, both a preconditioning step in 0.5 M sucrose–0.05 M Tris and a PEG treatment step were required for nonprotoplast transformation. The procedures described by Klebe et al. (11) for yeasts and *E. coli* were applied to *S. lactis* without success (data not shown).

The whole-cell protocol presented here was developed by modifying the procedure of Kondo and McKay (13) with respect to several parameters. The utilization of PEG as suggested by Wirth et al. (28) (i.e., PEG prepared in a succinate-based buffer and used at 0°C) seems important to the success of the whole-cell protocol described here. Transfection of LM0230 by the procedure of Kondo and McKay (13) omitting the enzyme treatment step did not result in the recovery of any transfectants (Table 2). However, Table 3 shows that PEG is not totally responsible for the failure to obtain transfectants with nonprotoplasted cells by the Kondo and McKay procedure. Substituting 30% PEG in SMMB at room temperature (14) for 35% PEG in TSCM at 0°C in the protocol presented here yielded transfectants, albeit at the lowest frequency. Transfectants may have been detected here and not in the experiment of Table 2 owing to the 1-h incubation at 37°C in SMMB used for the results shown in Table 3. Extended incubation (up to 1 h) in SMMB was subsequently shown to be important in obtaining transformants of whole cells.

Efforts to optimize the procedure identified several parameters important to whole-cell transformation. Transformation efficiency increased with increasing cell density (Fig. 2) up to late-log-phase cells. No efforts to differentiate the effect of cell density and growth phase of the cell have yet been done. However, the results indicate that a high density of log-phase cells results in the highest transformant yield obtained.

Another parameter important to transformation efficiency was incubation of the cells at 37°C in 0.5 M sucrose–0.01 M Tris, pH 7.0. Differences of almost 2 orders of magnitude in transformant recovery were found between 15 and 60 min of incubation at 37°C, suggesting that incubation in 0.5 M sucrose somehow acclimtes the cells to take up naked DNA. This acclimation to transformability is similar to that seen with *E. coli*, for which extended incubation in a cold $CaCl_2$ solution enables the cells to take up DNA (4).

As expected, the transformation levels and efficiency were dependent on the amounts of DNA added to the transformation reaction (data not shown). Levels of transformants increased with increased DNA levels, but transformation efficiency (transformants per microgram of DNA) was maximal at the lowest DNA levels tested. The specific DNA used also appeared to affect transformation frequency. The best recovery of transformants was seen with pSA3 and pAN50, a derivative of pSA3 containing the chloramphenicol acetyltransferase gene from pMH120-13 (M. C. Hudson and G. C. Stewart, manuscript in preparation) cloned at the *Bam*HI site. pVA856 and pTV1 DNAs transformed LM0230 at levels lower by approximately 10-fold. c2ϕ DNA transfection efficiency was quite low. However, this is probably because (i) the particular batch of DNA used appeared to be of poor quality and because (ii) transfection with c2ϕ DNA was only attempted early in the study, before optimization efforts. It is important, however, to note that all intact DNAs tested from *E. coli* or streptococcal origin were successfully and repeatably transformed.

The PEG treatment step was optimized relative to PEG molecular weight, concentration, and pH. It was found that 30% PEG 8000 at pH 6.5 gave optimal results. Treatment with PEG at cold temperatures as compared with room temperature also enhanced transformant recovery. Furthermore, PEG prepared in sucrose-based buffer was not as effective as PEG prepared in the succinate-based TSCM. The absolute requirement of PEG for the whole-cell transformation procedure was repeatedly demonstrated.

A heat shock (42°C for 5 min) was effective, raising transformant recovery twofold. Whether this functions to inactivate nucleases temporarily or confers other physiological effects aiding transformation is unknown.

Plating transformed cells into an osmotically stabilized medium was important to cell recovery, possibly resulting from some degree of osmotic sensitivity of cells after transformation. These data suggest that treatment of cells through the protocol presented here, even in the absence of intentional cell wall digestion, results in a degree of cell wall disruption, providing a means of DNA entry into the cell.

Transformation was also shown to be successful with frozen cells and DNA not purified through density gradients, albeit at lower efficiencies. These findings are significant in that these steps could represent significant time or cost savings or both for routine application of transformation. Studies determining the effect of increased time in frozen storage on transformation efficiency were not conducted.

The successful application of this transformation system to direct cloning techniques is important for the direct selection of clones in an *S. lactis* background. The cloning of the lactose-metabolizing genes directly into LM0230 was shown previously (13). The experiments reported here show a similar ability to subclone plasmid-encoded genes directly into LM0230. Since pSA3 was used here, no insertional inactivation marker was available for direct selection in *Streptococcus* species. Therefore, transformants were chosen at random for plasmid DNA evaluation. By choosing DNA concentrations that favored insertion (backbone/insert, 1:20), clones with inserts were recovered. These clones are currently being assayed for their phenotype in LM0230.

With the omission of the protoplasting step, transformation of *S. lactis* has proved to be more consistent and reliable in our hands. After optimization of this procedure, LM0230 transformants could be obtained at levels of 5×10^5 transformants per μg of DNA. Attempts to apply this optimized procedure to strains of *Streptococcus cremoris*, *Streptococcus thermophilus*, and other strains of *S. lactis*

have not yet been successful, suggesting a strain specificity of this particular procedure. In fact, preliminary evidence suggests that there is a difference in transformability of different isolates of LM0230. However, other lactic acid bacteria may be transformable via whole-cell procedures, but the conditions remain to be optimized. The empirical nature of this report precludes in depth conclusions regarding the mechanism of DNA uptake. The physiological state of the cell attained after incubation in sucrose has not been studied, but certainly holds a key to the effectiveness of this protocol. It is possible that this treatment promotes limited autolytic enzyme activity providing a means for the cell wall barrier to DNA uptake to be weakened. No experimental evidence for this possibility has been obtained. However, evidence for sucrose-induced autolysis has been demonstrated in other gram-positive bacteria (20). Additional experimentation in this area is warranted. Further investigation into the mechanism of whole-cell transformation could possibly lead to an understanding of the apparent strain specificity of this procedure and to the development of transformation systems for lactic acid bacteria nontransformable by protoplast means.

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LITERATURE CITED

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.
2. **Behnke, D., M. S. Gilmore, and J. J. Ferretti.** 1981. Plasmid pGB301, a new multiple resistant streptococcal cloning vehicle and its use in cloning of the gentamycin/kanamycin resistance determinant. Mol. Gen. Genet. **182**:414–421.
3. **Chopin, M.-C., A. Chopin, A. Rouault, and D. Simon.** 1986. Cloning in *Streptococcus lactis* of plasmid-mediated UV resistance and effect on prophage stability. Appl. Environ. Microbiol. **51**:233–237.
4. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1973. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
5. **Dao, M. L., and J. J. Ferretti.** 1985. *Streptococcus-Escherichia coli* shuttle vector pSA3 and its use in the cloning of streptococcal genes. Appl. Environ. Microbiol. **49**:115–119.
6. **Fornari, G. S., and S. Kaplan.** 1982. Genetic transformation of *Rhodopseudomonas sphaeroides* by plasmid DNA. J. Bacteriol. **152**:89–97.
7. **Fujita, Y., T. Okamoto, and R. Irie.** 1983. Protoplast formation and regeneration in lactic streptococci. Agric. Biol. Chem. **47**:2103–2105.
8. **Garcia, H. M., M. de los Angeles Martinez, R. Vazquez, and J. Delgado.** 1985. Transformation of *Kluyveromyces lactis* cells by plasmid DNA does not require alkaline cations. Biotechnol. Lett. **7**:723–726.
9. **Gasson, M. J., and P. H. Anderson.** 1985. High copy number plasmid vectors for use in lactic streptococci. FEMS Microbiol. Lett. **30**:193–196.
10. **Klaenhammer, T. R., L. L. McKay, and K. A. Baldwin.** 1978. Improved lysis of group N streptococci for isolation and rapid characterization of plasmid deoxyribonucleic acid. Appl. Environ. Microbiol. **35**:592–600.
11. **Klebe, R. J., J. V. Harriss, Z. D. Sharp, and M. G. Douglas.** 1983. A general method for the polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene **25**:333–341.
12. **Kok, J., J. M. van Dijl, J. M. B. M. van der Vossen, and G. Venema.** 1985. Cloning and expression of a *Streptococcus cremoris* proteinase in *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50**:94–101.
13. **Kondo, J. K., and L. L. McKay.** 1984. Plasmid transformation of *Streptococcus lactis* protoplasts: optimization and use in molecular cloning. Appl. Environ. Microbiol. **48**:252–259.
14. **Kondo, J. K., and L. L. McKay.** 1982. Mutanolysin for improved lysis and rapid protoplast formation in dairy streptococci. J. Dairy Sci. **65**:1428–1431.
15. **Kondo, J. K., and L. L. McKay.** 1982. Transformation of *Streptococcus lactis* protoplasts by plasmid DNA. Appl. Environ. Microbiol. **43**:1213–1215.
16. **Lee-Wickner, L.-J., and B. M. Chassy.** 1984. Production and regeneration of *Lactobacillus casei* protoplasts. Appl. Environ. Microbiol. **48**:994–1000.
17. **Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones.** 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. Gene **25**:145–150.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
20. **Ogata, S., K. H. Choi, and M. Hongo.** 1980. Morphological changes during conversion of *Clostridium saccharoperbutylacetonicum* to protoplasts by sucrose-induced autolysis. Microbiol. Immunol. **24**:393–400.
21. **Okamoto, T., Y. Fujita, and R. Irie.** 1983. Protoplast formation and regeneration of *Streptococcus lactis* cells. Agric. Biol. Chem. **47**:259–263.
22. **Sanders, M. E., and T. R. Klaenhammer.** 1981. Evidence for plasmid linkage of restriction and modification in *Streptococcus cremoris* KH. Appl. Environ. Microbiol. **42**:944–950.
23. **Sanders, M. E., P. J. Leonhard, W. D. Sing, and T. R. Klaenhammer.** 1986. Conjugal strategy for construction of fast acid-producing, bacteriophage-resistant lactic streptococci for use in dairy fermentations. Appl. Environ. Microbiol. **52**: 1001–1007.
24. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Methods for rapid plasmid DNA isolation, p. 147–148. *In* Experiments with gene fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Simon, D., A. Rouault, and M.-C. Chopin.** 1986. High-efficiency transformation of *Streptococcus lactis* protoplasts by plasmid DNA. Appl. Environ. Microbiol. **52**:394–395.
26. **Soutschek-Bauer, E., L. Hartl, and W. L. Saudenbauer.** 1985. Transformation of *Clostridium thermohydrosulfuricum* DSM-568 with plasmid DNA. Biotechnol. Lett. **7**:705–710.
27. **Takahashi, W., H. Yamagata, K. Yamaguchi, N. Tsukagoshi, and S. Udaka.** 1983. Genetic transformation of *Bacillus brevis* 47, a protein-secreting bacterium, by plasmid DNA. J. Bacteriol. **156**:1130–1134.
28. **Wirth, R., F. An, and D. B. Clewell.** 1986. High efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165**:831–836.
29. **Youngman, P. J., J. B. Perkins, and R. Losick.** 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917. Proc. Natl. Acad. Sci. USA **80**:2305–2309.
30. **Yu, R. S.-T., W. S. A. Kyle, A. A. Azad, and T. V. Hung.** 1984. Conditions for the production and regeneration of protoplasts from lactic streptococci. Milchwissenschaft **39**:136–139.
31. **Yu, R. S.-T., W. S. A. Kyle, T. V. Hung, and A. A. Azad.** 1984. Aspects of genetic transformation involving protoplasts and purified lac plasmid of *Streptococcus lactis*. Milchwissenschaft **39**:476–479.