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NOTES

Effect of Proteolytic Enzymes on Transfection and Transformation of *Streptococcus lactis* Protoplasts†

STEVEN A. WOSKOW‡ AND JEFFERY K. KONDO*
Department of Nutrition and Food Sciences, Utah State University, Logan, Utah 84322-8700

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With both chymotrypsin and mutanolysin used to form protoplasts, consistent transformation frequencies of 10^4 to 10^5 transformants and transfectants per μg of DNA were achieved. The procedure was used to transform protoplasts of *Streptococcus cremoris* CS224 at low frequency (5 transformants per μg of DNA).

A reliable and efficient procedure for introducing DNA into dairy starter cultures is essential for applying genetic engineering techniques to these industrially important bacteria. In 1982, polyethylene glycol (PEG)-induced transformation (12) of *Streptococcus lactis* and transfection (8) of *S. lactis* subsp. *diacetylactis* protoplasts were described. Since then, several protoplast transformation procedures have been developed for different strains of *S. lactis*, and low-frequency transformation of *Streptococcus cremoris* has been reported (18). Mutanolysin (12, 14), lysozyme (11, 19, 22), or combinations of both mutanolysin and lysozyme (7) have been used to form protoplasts for transformation. The efficiency of protoplast formation, regeneration, and transformation appear to be strain specific. Although many procedures have been published, transformation frequencies are variable, ranging from 10^2 to 10^6 transformants per μg of DNA.

Transformation of protoplasts generated from lysozyme treatment has been inefficient with our strains of *S. lactis* (unpublished results). Protoplasts formed from mutanolysin treatment are effective for transformation, but transformation frequencies may vary considerably depending on the particular commercial lot of mutanolysin (unpublished results). Commercial sources of this enzyme are known to contain different muralytic, proteolytic (17; Noel Brawn, Sigma Chemical Co., personal communication) and DNase activity (24). The proteolytic activity of different commercial lots of mutanolysin may vary from less than 0.01 to 0.04% of the lytic activity (Noel Brawn, Sigma Chemical Co., personal communication). This proteolytic activity is known to stimulate lytic activity of various enzymes (20), and it has been speculated that proteolytic enzymes enhance lytic activity by their clearing action against cell debris (26).

In this study the efficiency and repeatability of transformation and transfection could be improved by forming protoplasts with mutanolysin in conjunction with proteolytic enzymes. By optimizing various parameters, we developed a method which consistently results in 10^4 to 10^5 transformants and transfectants per μg of DNA when using protoplasts from *S. lactis* LM2301. We have also used this method to transform *S. cremoris* CS224 at low frequencies.

Bacterial strains, bacteriophages, and plasmids. *S. lactis* LM2301, a plasmid-cured derivative of *S. lactis* C2 (5), was used as a recipient for transformation and transfection experiments. *S. cremoris* CS224, received from William Sandine (Department of Microbiology, Oregon State University, Corvallis) contains seven plasmids with masses of 57, 35.8, 27, 26, 20.5, 16.8, and 1.8 megadaltons (MDa). Cultures were maintained by biweekly transfer at 30°C in M17 broth (21) containing glucose (M17G) or lactose (M17L). *S. lactis* JK301 (14), which contains pGB301 (2, 3), was used to isolate plasmid DNA for transformation experiments. Plasmid pGB301 is a 6.5-MDa *Streptococcus sanguis* cloning vector coding for MLS (macrolide, lincosamide, streptogramin B) and chloramphenicol resistance. *S. lactis* C2 lytic bacteriophage c2 (16) was propagated by infection of *S. lactis* C2 in M17 broth.

DNA isolation procedures. Bacteriophage was isolated by infecting *S. lactis* C2 with c2 bacteriophage and incubating at 30°C until complete lysis was observed (3 to 5 h). Bacteriophages were PEG concentrated by the method of Yamamoto and Alberts (25), and DNA was extracted and purified as previously described (8). Plasmid DNA was isolated by the method of Anderson and McKay (1). Plasmids used for transformation were further purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation (10). Desalting was performed by repeated ultrafiltration in a Centricon 30 microconcentrator (Amicon Division, W.R. Grace & Co., Danvers, Mass.). Plasmids and bacteriophage DNA were visualized by electrophoresing DNA samples through a 0.6% horizontal agarose gel in TBE buffer (0.089 M Tris hydrochloride, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) for 4 h at 6 V/cm. DNA concentration was determined spectrophotometrically (absorbance at 260 nm/absorbance at 280 nm).

Protoplast formation. The final procedure for formation of protoplasts of *S. lactis* LM2301 was performed as described previously (14) with modifications. A 1% inoculum of an active M17G broth culture was made into 30 ml of M17G broth prewarmed to 30°C. Cells were grown for 4 or 16 h overnight at 30°C (2.5×10^9 CFU/ml), centrifuged at 7,600 ×

* Corresponding author.
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‡ Present address: Department of Food Technology, Iowa State University, Ames, IA 50011-1060.

TABLE 1. Transformation and transfection of mutanolysin- and chymotrypsin-treated cells of *S. lactis* LM2301[a]

Mutanolysin (U/ml)	Chymotrypsin (U/ml)	No. of transfectants/ μg of DNA	No. of transformants/ μg of DNA
25	0	3.1×10^2	2.9×10^1
25	1.25	4.6×10^3	2.3×10^2
25	6.25	1.3×10^3	2.3×10^2
25	12.50	1.9×10^3	9.8×10^1
25	25.00	9.3×10^2	1.1×10^2

[a] *S. lactis* LM2301 cells were grown for 3 h (OD_{600}, 0.65) and treated with mutanolysin for 30 min, followed by chymotrypsin treatment for 15 min. Conditions for transformation include 1 μg of pGB301 DNA, a 20-min PEG treatment time, and selection of Cm^r transformants by the overlay technique.

g for 5 min, washed in cold (4°C) double-deionized water, and suspended in 7.6 ml of 0.5 M sucrose in 0.01 M Tris hydrochloride, pH 7.0. Mutanolysin (Sigma) was added to a final concentration of 25 U/ml, followed immediately by addition of chymotrypsin (Boehringer Mannheim, Indianapolis, Ind.) to a 1.25-U/ml final concentration. The cell suspension (8 ml, final volume) was incubated at 37°C for 15 min, centrifuged at 2,000 × *g* for 10 min, washed in 5 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 suspended in 1.0 ml of SMMB. Resuspension of protoplasts was performed in an orbit environ-shaker (Lab-Line Instruments, Inc., Melrose Park, Ill.) on a setting of 200 rpm at 30°C.

Transformation. Transformation experiments were performed as previously described (14) with modifications in plating methods. A 0.1-ml portion of an appropriate dilution of transformed cells was added to 5 ml of M17G top agar (0.5% agar) containing 0.5 M sucrose as an osmotic stabilizer (SM17G), gently mixed, and overlaid on SM17G bottom agar (1.5% agar) containing 5 μg of chloramphenicol per ml. Later, cells were plated by the spread plate technique onto SM17G bottom agar supplemented with 5 μg of chloramphenicol per ml. Transformants were scored after 7 days of incubation at 30°C. Protoplast concentration was kept constant for each transformation trial by pooling protoplast suspensions before distributing them for transformation. SMMB buffer was filtered through a 0.45-μm membrane filter before use, and all other reagents and buffers were autoclaved and quickly cooled in a water bath. Values for all transformation experiments were from two or more independent trials. No transformants were observed when cells were incubated with chymotrypsin alone or when DNase I (Sigma) was added to plasmid preparations before transformation.

Transfection. Transfection was performed essentially in the same way as transformation. Appropriate dilutions of the transfected protoplasts were made in SMMB buffer and plated by adding 0.1 ml of protoplasts to 3 ml of SM17G top agar (0.5% agar) containing 0.1 ml of 1 M $CaCl_2$ and 0.2 ml of indicator cells (*S. lactis* LM2301). PFU were counted after 24 h of incubation at 30°C. Treatment of bacteriophage DNA with DNase I inhibited transfection. No plaques appeared when bacteriophage DNA was not added and the DNA used for transfection was free of PFU in conventional phage assays.

Effect of chymotrypsin, pronase, and trypsin on transformation frequency. Preliminary experiments indicated that chymotrypsin treatment of cells during protoplast formation with mutanolysin enhanced transformation and transfection of *S. lactis* LM2301 (Table 1). Parameters affecting transformation and conditions necessary for optimal transformation frequencies were then examined.

Protoplasts of *S. lactis* were prepared for transformation by treating cells with various concentrations of mutanolysin and chymotrypsin and incubating for different time periods (Table 2). Incubation for 15 min increased transformation frequencies but only when 1.25 or 6.25 U of chymotrypsin per ml was used. After 30 min of incubation, only the lowest concentrations of the two enzymes increased transformation efficiency. Formation of protoplasts with mutanolysin (25 U/ml), chymotrypsin (1.25 U/ml), and an incubation time of 15 min was found to be optimal in further experiments (data not shown).

Pronase and trypsin were compared with chymotrypsin to assess the effect of different proteases on transformation frequency. Chymotrypsin (3.1×10^4 transformants per μg of DNA) and trypsin (2.8×10^4 transformants per μg of DNA) increased the transformation frequency approximately 100-fold over that with mutanolysin used alone (5.1×10^2 transformants per μg of DNA), while pronase only increased transformation frequency to 1.3×10^3 transformants per μg of DNA.

Effect of cell growth stage. Transformation appears to be dependent on cell growth phase and on the extent to which cell wall material is removed from the cells (19). To examine the effect of cell growth stage, protoplasts were formed from *S. lactis* cultures grown for 1, 2, 3, 4, or 5 h. Cell densities were adjusted to approximately 5×10^9 CFU/ml (1.2 OD_{600} units) for each time period prior to protoplast formation and transformation. Transformation was performed as described in Materials and Methods and plated by the soft agar overlay technique. There was a sharp decrease in transformation frequency with protoplasts prepared from 2-h cultures (optical density of 0.15 at 600 nm), followed by an increase in transformation frequency to 2.0×10^4 transformants per μg of DNA with protoplasts prepared from 4-h cultures (optical density of 1.2 at 600 nm) (Fig. 1). Transformation frequencies for protoplasts prepared from 4-h cultures treated with mutanolysin only (6.7×10^3 transformants per μg of DNA) were lower than when mutanolysin plus chymotrypsin was used.

Protoplasts were then formed from *S. lactis* cultures grown for 2, 4, 6, and 16 h (overnight). Cell density was adjusted to an OD_{600} of 1.2 for each time period prior to protoplast formation, and transformation was performed as

TABLE 2. Effect of mutanolysin and chymotrypsin concentrations and time of incubation on transformation frequencies of *S. lactis* LM2301[a]

Enzyme concn (U/ml)		Growth (CFU/μg of DNA) after treatment for:	
Mutanolysin	Chymotrypsin	15 min	30 min
25.0	0	6.2×10^2	3.9×10^3
12.5	1.25	1.2×10^4	1.2×10^4
12.5	6.25	3.8×10^3	2.0×10^3
12.5	12.5	9.0×10^2	3.6×10^2
25.0	1.25	1.1×10^4	8.4×10^3
25.0	6.25	5.2×10^3	1.5×10^2
25.0	12.5	3.5×10^2	2.9×10^2
50.0	1.25	1.2×10^3	1.9×10^2
50.0	6.25	1.0×10^3	1.7×10^2
50.0	12.5	1.4×10^2	3.6×10^2

[a] Protoplasts were formed from 3-h cultures (OD_{600}, 0.65) by adding different concentrations of mutanolysin plus chymotrypsin and incubating for either 15 or 30 min. One microgram of pGB301 was used, and transformed protoplasts were plated with soft agar overlays.

before except that transformed protoplasts were spread-plated instead of overlaid. The 16-h cultures (time zero) were transformed optimally (ca. 7.0×10^4 transformants per μg of DNA). Transformation frequencies were lower when 16-h cultures were treated with mutanolysin only (4.5×10^3 transformants per μg of DNA). Transformation frequencies at 2 h showed greater variability (3.7×10^4 to 2.8×10^3 transformants per μg of DNA) when protoplasts were spread-plated, which is similar to the findings of Kondo and McKay (14).

Transfection of protoplasts prepared from cultures grown for 2, 4, 6, and 16 h showed optimal frequencies when protoplasts were formed from 16-h (2.0×10^5 transfectants per μg of DNA) and 4-h (1.0×10^5 transfectants per μg of DNA) cultures. Transfection frequencies were lower when protoplasts were prepared from 2-h (4.7×10^4 transfectants per μg of DNA) and 6-h (5.3×10^4 transfectants per μg of DNA), but the decrease in frequencies at those times was not as pronounced as the decrease in transformation frequencies. Transfection frequencies with mutanolysin alone were lower for both 4-h (4.2×10^3 transfectants per μg of DNA) and 16-h (3.0×10^3 transfectants per μg of DNA) cultures.

Cell lysis. The rate of production of osmotically fragile cells during treatment with mutanolysin and chymotrypsin was examined. Cultures were grown for 1, 2, 3, 4, or 16 h and suspended in 0.01 M Tris hydrochloride (pH 7.0) without osmotic stabilizer. Cell lysis was followed by monitoring optical density over time. The 2-h cultures were more susceptible to lysis than 4- or 16-h cultures (Fig. 2). When 4-h cultures were treated with both enzymes, there was greater and more rapid production of osmotically fragile cells than when 4-h cultures were treated with mutanolysin alone.

Transformation of osmotically stable cells. Transformation of osmotically stable cells, cells which have not formed protoplasts and thus are resistant to osmotic shock, was performed to determine the amount of cell wall removal necessary for DNA uptake. Osmotically stable cells were isolated after protoplast formation by dilution in sterile water and transformed as described in Materials and Methods except that cells were plated on M17G containing chloramphenicol (5 μg/ml). No transformation of osmotically stable cells was observed, whereas 5.0×10^4 transformants per μg of DNA were obtained with protoplasts. The fraction of protoplasts able to transform was only 0.002% of the viable protoplasts, which results in a transformation efficiency of 1.7×10^{-5} transformants per viable protoplast.

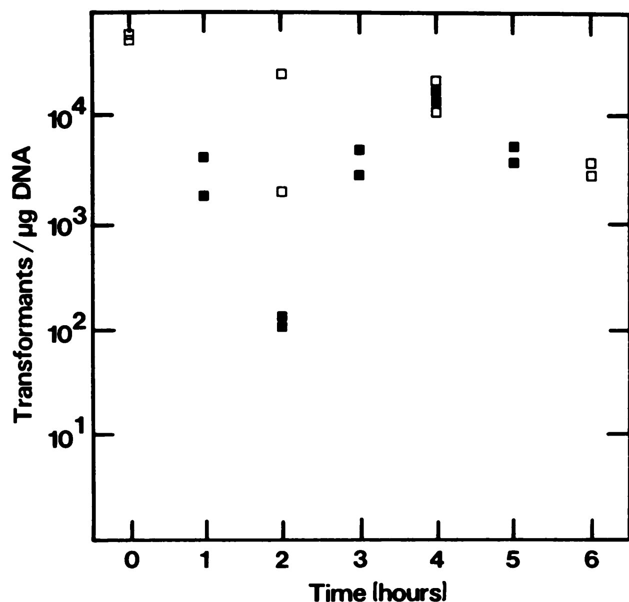

FIG. 1. Effect of growth stage of cultures used to form protoplasts on transformation frequency. Protoplasts of *S. lactis* LM2301 were prepared from cultures taken after 1, 2, 3, 4, 5, 6, and 16 h (time zero) of incubation. Protoplasts were transformed as described in Materials and Methods and plated by either the overlay technique (■) or spread plating (□).

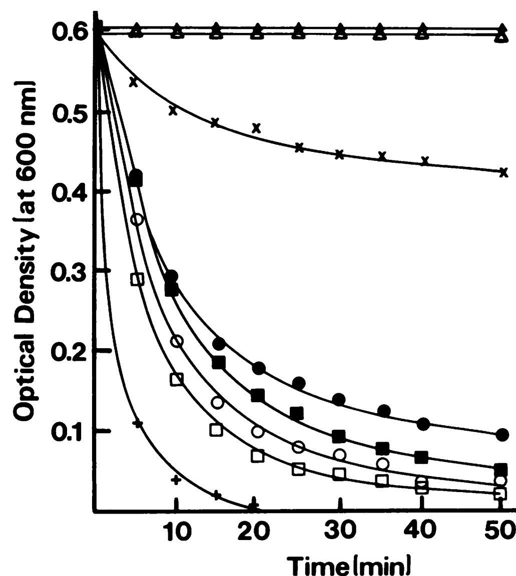

FIG. 2. Time course for the production of osmotically fragile cells of *S. lactis* LM2301. Cells were incubated for various time periods and suspended in hypotonic buffer. Mutanolysin and chymotrypsin were added, and optical density at 600 nm was monitored. Controls: △, 4-h cultures treated with chymotrypsin only; ▲, 4-h cultures with no enzyme treatment. Symbols: ■, 1 h of growth treatment with both enzymes; +, 2-h growth treatment with both enzymes; □, 3-h growth treatment with both enzymes; ○, 4-h growth treatment with both enzymes; ●, 4-h growth treatment with mutanolysin only; ×, 16-h growth treatment with both enzymes.

Effect of osmotic stabilizers and plating method. Succinate has been used as an osmotic stabilizer in medium used for regeneration of *S. faecalis* protoplasts (24) and may be effective in increasing transformation frequencies in *S. lactis* (L. L. McKay, personal communication). To test the effect of plating method and the influence of different osmotic stabilizers, transformants were either spread-plated or overlaid onto M17G agar supplemented with either 0.5 M sucrose (SM17G) or 0.25 M succinate (SaM17G). Transformation frequencies of 10^5 transformants per μg of DNA could be achieved by overlaying or spread-plating onto SaM17G. However, more consistent results were obtained when protoplasts were spread-plated onto SM17G (2.5×10^5 transformants per μg of DNA).

Effect of PEG-DNA treatment time for induction of transformation. The time of PEG-DNA treatment of protoplasts has been shown to affect transformation frequency. PEG treatment times of 2 (19), 5 (22), and 20 (7, 14) min have been reported for optimal transformation of different strains of *S. lactis*, and a 10-min PEG-DNA treatment time is used for transfection of *S. lactis* subsp. *diacetylactis* (8). To examine

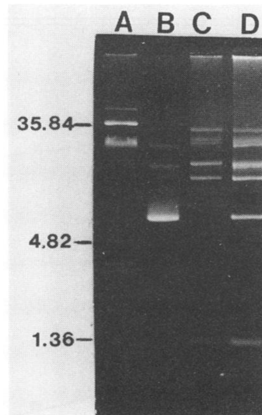

FIG. 3. Agarose gel electrophoresis of plasmid DNA of parental and transformed strains. Lanes: A, *Escherichia coli* V517 reference plasmids; B, pGB301; C, *S. cremoris* CS224 parental strain used as a recipient for transformation; D, *S. cremoris* SW301, a Cm^r transformant of *S. cremoris* CS224 containing pGB301. Plasmid sizes (in megadaltons) are indicated.

the effect of PEG-DNA treatment time, protoplasts were treated with PEG and DNA for 2, 5, 10, and 20 min. We found that maximal transformation frequencies were achieved after a 20-min PEG-DNA treatment time, although shorter times could still be used. These results agree with those of Kondo and McKay (14).

Transformation of *S. cremoris*. The procedure described was used to transform *S. cremoris* to Cm^r with plasmid pGB301 at low frequencies (5 transformants per μg of DNA). Acquisition of pGB301 was suggested by the Cm^r phenotype and was confirmed by agarose gel electrophoresis of plasmid DNA isolated from transformants. All transformants (designated *S. cremoris* SW301) contained the normal complement of plasmids plus a newly acquired plasmid of approximately 6.5 MDa which comigrated with pGB301 (Fig. 3). Therefore, it may be possible to develop high-efficiency transformation of *S. cremoris* with pGB301 since it is able to replicate and express its genes in this strain.

It is unknown why proteolytic enzymes enhance transformation, but it appears that DNA uptake rather than regeneration of protoplasts is affected since regeneration-independent transfection frequencies are also increased. If proteases act by clearing cell debris (26), it is possible that observed increases in transformation frequencies are due to more efficient protoplast formation or that DNA is more accessible to protoplasts.

Proteases may also degrade cell wall and cell membrane proteins which inhibit transformation and transfection. Hurst and Stubbs (9) reported the appearance of holes in the cell wall of *S. lactis* which are blocked by trypsin-sensitive conical protrusions. Removal of conical protrusions by proteases may open the holes and allow better DNA uptake. Other cell wall and cell membrane proteins which bind DNA and make it unavailable for uptake by protoplasts may be degraded by proteases. DNases secreted during protoplast formation may also decrease transformation and transfection frequencies (15). Using the DNase plate assay (Difco Laboratories, Detroit, Mich.), we observed extracellular DNase activity only after formation of protoplasts (unpublished results). It is possible that protease treatment during protoplast formation is able to degrade transformation-inhibiting DNases.

Using lysozyme to form protoplasts, Simon et al. (19) found that optimal transformation was achieved from early-log-phase (2-h) cultures. These differences may reflect the amount of cell wall removed during protoplast formation. The streptococcal cell wall is composed of polysaccharide and trypsin-sensitive mucopeptides which are present at greater amounts in stationary-phase cultures (23). The susceptibility of streptococcal cells to the action of either enzyme is dependent on the age of the culture prior to the enzyme treatment (4, 6). Also, mutanolysin has been shown to be more active in degrading cell walls of *S. lactis* (13). The weaker activity of lysozyme and the susceptibility of cell walls during early log phase may explain why 2-h cultures treated with lysozyme are transformed optimally, whereas optimal transformation with mutanolysin- and chymotrypsin-treated cells requires longer growth periods. Therefore, it appears that there is a critical amount of cell wall which must be removed for optimal transformation. Simon et al. (19) have demonstrated that optimal transformation frequencies are obtained when there is limited cell wall digestion. However, we have found that enough cell wall must be removed to render cells osmotically sensitive.

The transformation procedure reported here has eliminated much of the variability that we have observed in transformation frequencies. The proteolytic activity of commercial sources of mutanolysin may be a significant factor affecting the variability of transformation frequencies. By careful reagent preparation and handling of protoplasts and formation of protoplasts with a combination of mutanolysin and chymotrypsin, transformation frequencies have been consistent. We also have not had to readjust enzyme concentrations and treatment times with each new lot of mutanolysin. However, the procedure is still strain dependent, and conditions for transformation of new strains may have to be optimized.

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