

High-Efficiency Transformation of *Streptococcus lactis* Protoplasts by Plasmid DNA

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***Streptococcus lactis* IL1403 protoplasts were transformed by plasmid pIL204 (5.5 kilobases), which conferred erythromycin resistance with an average efficiency of 5×10^6 transformants per μg of supercoiled DNA. The procedure used and transformation efficiencies obtained were close to those described for *Bacillus subtilis* (G. Chang and S. N. Cohen, Mol. Gen. Genet. 168:111-115, 1979).**

The group N streptococci (*Streptococcus lactis*, *S. cremoris* and *S. lactis* subsp. *diacetylactis*) include strains of industrial importance largely used in dairy fermentations. The need for recombinant DNA techniques in studying the molecular biology of these bacteria and constructing improved strains has been emphasized previously (4, 7, 11). These techniques rely on the introduction of recombinant DNA into the cells. Transformation of *S. lactis* protoplasts by plasmid DNA, but with a very low efficiency, was first described by Kondo and McKay (9). Later, the same authors examined several parameters affecting protoplast transformation and increased the transformation efficiency to 5×10^3 to 4×10^4 transformants per μg of DNA (10). Other researchers have changed some parameters (13) without improving the transformation efficiencies reported by Kondo and McKay (10).

This range of transformation efficiencies allowed the direct cloning of plasmid genes into *S. lactis* (3, 10). However, the development of convenient in vitro recombinant DNA techniques for *S. lactis* still relies on a reliable and highly efficient transformation technique, especially when recovery of rare recombinant classes is critical. The need for such a technique was recently emphasized by the difficulties encountered in using shuttle-cloning strategies. Cloning of lactose genes from *S. lactis* in *S. sanguis* led to deletions and integration into the chromosome (5). Cloning of a *S. cremoris* proteinase in *Bacillus subtilis* and subsequent transfer and expression in *S. lactis* has been straightforward, but cloning in *Escherichia coli* was not successful, probably because the expression of this gene was lethal in *E. coli* (8). In this paper we describe a technique for the transformation of *S. lactis* protoplasts which consistently leads to an average efficiency of 5×10^6 transformants per μg of supercoiled plasmid DNA. This procedure is very close to that previously described by Chang and Cohen for *B. subtilis* (1).

The plasmid-free strain IL1403 of *S. lactis* lacking restriction-modification system (2) was used as recipient in all transformation experiments. It was grown in M17 medium (12) in which lactose was replaced by glucose (M17glc). Most experiments were performed with plasmid pIL204 (5.5 kilobases [kb]) conferring erythromycin resistance (Em^r) and

deriving from pHV1301 (3) by in vitro deletions. This plasmid will be described in detail elsewhere.

A 20-ml sample of an early log-phase culture (ca. 2×10^8 CFU/ml) grown in M17glc was washed in distilled water. The culture was then harvested and suspended in 5 ml of SMM-M17glc, which was prepared by mixing equal volumes of $4 \times$ M17glc broth and $2 \times$ SMM buffer (14). Lysozyme (Aflact; Sochal, Levallois-Perret, France) was added to 10 mg/ml final concentration. The suspension was incubated for 2 h at 37°C . The protoplasts were pelleted at $3,000 \times g$ for 10 min and suspended in 5 ml of SMM-M17glc. They were pelleted again and resuspended in 650 μl of SMM-M17glc. Then 50 μl of plasmid DNA in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]) mixed with 50 μl of $2 \times$ strength SMM solution was added, followed by 2 ml of a polyethylene glycol (molecular weight, 2,700 to 3,300; E. Merck AG, Darmstadt, Federal Republic of Germany) solution (40%, wt/vol). After a 2-min exposure to polyethylene glycol, protoplasts were diluted by addition of 6.5 ml of SMM-M17glc, pelleted at $3,000 \times g$ for 10 min and suspended in 1.3 ml of M17glc supplemented with sucrose (0.5 M). To allow phenotypic expression of plasmid-encoded Em^r , protoplasts were incubated for 1.5 h at 30°C . Transformants were recovered by pour-plating into M17glc agar (1.2% Bacto-Agar; Difco Laboratories, Detroit, Mich.) supplemented with sucrose (0.5 M) and erythromycin (2.5 $\mu\text{g}/\text{ml}$) and incubated for 5 to 7 days at 30°C . No transformants were observed when pancreatic DNase (0.03 $\mu\text{g}/\text{ml}$) was added prior to polyethylene glycol treatment.

The transformation efficiency was highly dependent on the growth phase of the cells to be converted to protoplasts and on the extent to which cell walls had been removed from the protoplasts. Optimum results were obtained with protoplasts prepared from an early-log-phase culture, with an optical density at 660 nm ranging from 0.10 to 0.40 (Fig. 1). Use of mid-log-phase culture decreased the efficiency of transformation by 1 order of magnitude, although the number of available protoplasts was higher and the number of osmotically stable cells was unchanged (data not shown). Optimum results were also obtained when the protoplasts were prepared with lysozyme concentrations ranging from 1 to 40 mg/ml, although higher lysozyme concentrations did not hamper protoplast regeneration. In addition, the replacement of SMM-M17glc medium by SMM buffer for protoplast production drastically reduced the transformation efficiency.

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The transformation efficiency showed a linear relationship to DNA concentration over a 10^4 -fold range (10 to 10^{-3} μg of pIL204 plasmid DNA). This indicated that the number of plasmid DNA molecules needed to yield one transformed cell remained constant over this range of DNA concentrations. In our case, 2.3×10^4 molecules of plasmid pIL204 were needed to obtain one *S. lactis* transformed protoplast, which is close to the number of molecules of plasmid pC194 (1.6×10^4) needed to obtain one *B. subtilis* transformed protoplast (1).

Three related plasmids differing in size, pAM β 1 (26.5 kb), pHV1301 (13 kb), and pIL204 (5.5 kb), were used to transform *S. lactis* protoplasts (Table 1), and a marked decrease in transformation efficiency was observed with larger plasmids. This effect was identical to that observed by Imanaka et al. in the transformation of *B. stearothermophilus* protoplasts (6) but was more pronounced than that observed by Kondo and McKay (10).

The high-efficiency transformation reported here could result from a high transformability of the *S. lactis* strain used throughout the study. However, this seems unlikely because no more than 10^4 transformants per μg of DNA could be obtained when this strain was transformed with plasmid DNA by the procedure described by Kondo and McKay (10).

The procedure described here consistently yielded a transformation efficiency 100-fold higher than the maximum efficiency previously reported by using the procedure described by Kondo and McKay (10). This might be explained by differences in protoplast formation and regeneration. First, we have shown that limited cell wall digestion is required for optimum protoplast transformation efficiency. It is likely that the mutanolysin treatment described by Kondo and McKay (10) leads to more extensive cell wall digestion, which may unfavorably affect the transformation efficiency. Second, under the lysozyme treatment conditions we used, without accounting for chaining of whole bacteria, 100 to 200% of protoplasts regenerated. This was much higher than

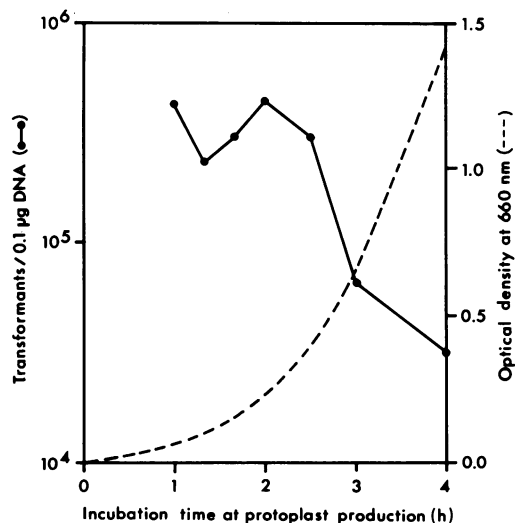


FIG. 1. Effect on transformation efficiency of the growth phase of the culture used to prepare protoplasts. Protoplasts of *S. lactis* IL1403 were prepared from aliquots of a culture taken after various incubation times and transformed with 0.1 μg of plasmid pIL204 as described in the text. The optical density at 660 nm of the culture used to prepare the protoplasts was continuously recorded.

TABLE 1. Effect of plasmid size on transformation efficiency of *S. lactis* protoplasts^a

Plasmid	Plasmid size (kb)	No. of transformants/ μg of DNA	No. of DNA molecules needed to yield one transformant
pAM β 1	26.5	7.8×10^4	4.5×10^5
pHV1301	13.0	1.0×10^6	6.9×10^4
pIL204	5.5	6.9×10^6	2.3×10^4

^a *S. lactis* IL1403 protoplasts were transformed with 1 μg of each plasmid DNA as described in the text.

the 3% regeneration percentage observed by Kondo and McKay after mutanolysin treatment (10) and may also account for an increased transformation efficiency.

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