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Successive Protoplast Transformation of *Bacillus subtilis* by Plasmid DNA under Low Concentration of Lysozyme

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A protoplast transformation system for *Bacillus subtilis* AC819 with pUB110 plasmid DNA under high pressure of kanamycin (100 μg/ml) has been developed by using extremely low concentration of lysozyme, 2.5 μg/ml, with a high regeneration efficiency of protoplasts. The optimum transformation of protoplasts was achieved under the conditions as follows : lysozyme concentration, 2.5μg/ml ; incubation period with lysozyme at 40.5 C, for 20 min ; intermediate cultivation, at 30 C for 90 min without shaking ; incubation temperature for regeneration, at 30 C. A response of kanamycin-resistant transformants to the number of protoplasts reached 1.9 x 10^4 per 370 ng of plasmid DNA, which corresponds to 100% per regenerants, and its efficiency was 10^3 fold higher than that of competent cell transformation.

INTRODUCTION

Protoplast transformation was established by Chang and Cohen (1979), and a gene transfer system in bacilli has been well documented (Brown and Carlton, 1980 ; Vorobjeva *et al.*, 1980 ; Imanaka *et al., 1981* ; Martin *et al.*, 1981). Successful protoplast transformation of bacilli requires at least both efficient utilization of DNA in the transformation process and efficient regeneration of protoplasts. We found the most limiting step in this system was the regeneration of protoplasted cells, which occurs the formation of L-form colonies and subsequently the regeneration of those L-form colonies to normal bacilli-form cells (Akamatsu and Sekiguchi, 1989). In these steps, however, surface of protoplasted cells generated with lysozyme might have an influence on the regeneration efficiency of protoplasts. Lysozyme was used for protoplast formation at varied concentrations as follows ;2 mg/ml (Chang and Cohen, 1979), 20 mg/ml (Bakhiet and Stahly, 1985) in *Bacillus subtilis*, 2 mg/ml in *B. licheniformis* (Jensen and Hulett, 1989), and 1 mg/ml in *B. megaterium* (Tatsubo *et al.*, 1990). After 20 min with lysozyme, cell walls of bacilli were completely removed under such a high enzyme concentration, and the protoplasted cells were appeared. It is, therefore, necessary for the protoplasted cells to be overlaid with the top agar for obtaining a high frequency of regeneration. The most efficient transformation systems were

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established for strains of *B. stearothermophilus* (Imanaka *et al.*, 1982 ; Liao *et al.*, 1986). In these transformation systems, protoplasts of *B. stearothermophilus* were prepared with the final concentration of 1 μg/ml in order to improve regeneration efficiency.

We attempted to improve the regeneration efficiency of protoplasts, which were prepared with a low concentration of lysozyme, and to achieve the high frequency transformation of *B. subtilis* with a low DNA concentration. In this report, we describe the development of a protoplast transformation system for *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and **plasmid**

Bacillus subtilis strain AC819 (*hisH, strA, tet-1, smo-1)* was used for all transformation procedures. Plasmid pUB110 carrying a kanamycin (Km)-resistant gene was used for transformation.

Media and materials

For bacterial growth, Penassay antibiotics medium 3 (PAB, Difco) was used. Sucrose-magnesium-malate (SMM) buffer of Wyrick and Rogers (1973) was the same as follows ; SMM buffer contained 0.5 M sucrose, 0.02 M maleic acid (pH6.5) and 0.02 M $MgCl_2$. For protoplast regeneration, Trypticase Soy Broth (TSB, BBL) medium containing 3% TSB, 0.02 M $MgCl_2$, 0.3 M sodium succinate (pH 7.3), 0.1 wt% bovine serum albumin (Nacalai Tesque, Inc., Kyoto, Japan) and 2% Bacto agar (Difco) was used with or without 100 μg/ml of kanamycin.

Preparation of **plasmid** DNA

Plasmid DNA was prepared by CsCl-ethidium bromide equilibrium density gradient centrifugation as described previously (Hara *et al.*, 1983).

Protoplasts Transformation

(i) Protoplast formation

Protoplasts were prepared according to the slightly modified method described by Akamatsu and Sekiguchi (1983). A 0.1 ml of preculture of *B. subtilis* AC819 was inoculated into 25 ml of PAB medium in an Erlenmyer flask to cultivate the bacterial cells at 37 C for 3 hr with shaking. The culture was centrifuged (5,000 rpm at 4 C, for 2 min) and the bacteria'were suspended in 4 ml of SMM supplemented with 2.5 mg per ml of lysozyme (Sigma Chem. Co.). The mixture was gently shaken (80 strokes per min) in 50 ml Erlenmyer flask at 40.5 C for 20 min, after that added 10 ml of SMM, centrifuged (5,000 rpm at 4 C for 3 min) and resuspended in 500 μl of SMM buffer.

(ii) Polyethylene glycol (PEG)-induced transformation

A 250 μl of the protoplast suspension, after adding plasmid DNA pUB110, was gently added 750 μl of 80% polyethylene glycol (# 4000, Nakarai Chemical Co., Kyoto). The mixture was kept on ice for 2 min, and then vigorously added 5 ml of SMM, further added 15 ml of SMM and centrifuged (5,000 rpm at 4 C for 5 min).

(iii) Phenotypic expression

The pellet suspended in 1 ml of TSB medium was incubated at 30 C for 90 min without shaking.

(iv) Regeneration of protoplasts and selection of transformants

A 100 μl volume of the protoplasts sample was spread onto TSB 2%-agar plate containing kanamycin (100 μg/ml) for direct selection of transformants without overlaying. Transformation frequency was scored after incubation of the protoplasts at 30 C for 2 days.

Competent cells transformation

Competent cells were prepared as described by Bott and Wilson (1967) and transformation was carried out as described previously (Aumayr et al., 1981).

RESULTS AND DISCUSSION

Optimal conditions for protoplast transformation

The protoplast transformation procedures established for *B. subtilis* (Chang and Cohen, 1979) were applicable for *B. licheniformis* (Jensen and Hulett, 1989) and *B. megaterium* (Takubo et al., 1990). Imanaka *et al. (1982)* was succeeded to achieve the protoplast transformation for thermophilic bacilli with a low lysozyme concentration. In order to further maximize the efficiency of our transformation procedure, we

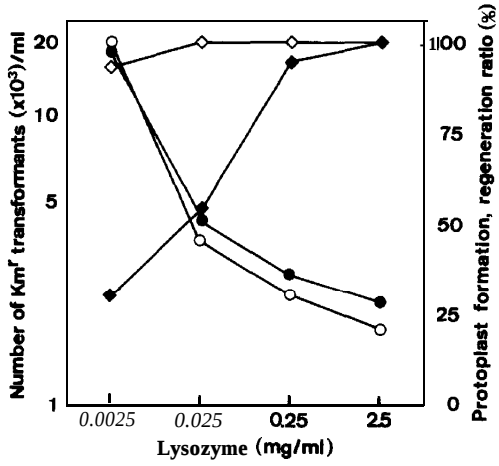

Fig. **1.** Effect of lysozyme concentration on protoplast transformation.

Culture was treated with various concentrations of lysozyme for preparation of protoplasts at 40.5 C for 20 min. The protoplasts contacted with pUB110 in polyethylene glycol were incubated at 30 C for 90 min without shaking for phenotypic expression, and spread onto TSB agar plates with and without kanamycin. The colonies regenerated on TSB agar plates containing kanamycin were counted as Km' transformants. Number of protoplasts were estimated as round-shape cells with microscopic observation or by taking the number of colonies regenerated on sodium succinate- and $MgCl_2$-free TSB agar plates after adding water from the number of colonies grown on complete TSB agar plates. Symbols: ●, number of Km' transformants ; ○, regeneration ratio ; ◆, protoplast formation ratio with microscopic observation; ◇, protoplast formation ratio by plating on TSB agar plate.

investigated the effects of various concentrations of lysozyme on protoplast transformation (Fig. 1). The number of protoplasts were estimated by taking the number of colonies regenerated on sodium succinate- and $MgCl_2$-free TSB agar plates after adding water from the number of colonies grown on a complete TSB agar plates. As shown in Fig. 1, at 2.5 μg/ml of lysozyme, the highest efficiency of protoplast transformation was achieved and 1.9 $x\,10^4$-Km^r transformants were obtained. The significant difference in protoplast formation ratio was not detected in the series of lysozyme concentrations used, whereas a regeneration ratio of protoplasts was greatly differred depend on used lysozyme concentration. The lower concentration of lysozyme is, the higher the regeneration ratio of protoplasts under the same experimental conditions. The number of regenerated colonies from protoplasts treated with 2.5 μg/ml of lysozyme was 1.9×10^4 per ml, which corresponds to 100% as a regeneration ratio. The high efficiency of regeneration of protoplasts might be resulted from the states of surface of protoplasted cells treated with lysozyme. It seems that some remained pieces of cell wall might act as a primer for the cell wall synthesis on regeneration of protoplasted cells. Subsequently, a higher efficiency of protoplast transformation might be achieved. In fact, the use of 2.5 μg/ml of lysozyme let to form less than 35 % as a round-shape bacilli by microscopic observation.

Figure 2 shows the effects of DNA concentrations on protoplast transformation. The transformation ability at different concentrations of plasmid DNA for *B. subtilis* shows a sensitivity potentially capable of detecting less than 37 pg of DNA. The relatively high transformation efficiency, 3 X 10^3 Km' transformants, of protoplasted cells with a low concentration of lysozyme, 2.5 μg/ml, was achieved with extremely lower DNA concentration. It is noted that, furthermore, the transformation efficiency of protoplasted cells with 2.5 μg/ml of lysozyme was much higher than that with 2.5

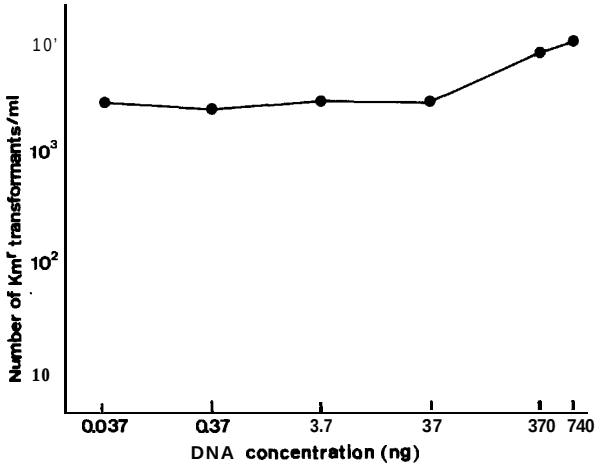

Fig. 2. Effect of DNA concentration on transformation efficiency.

Protoplasts were transformed with 370 ng of pUB110 as described above, and 0.1 ml of aliquots were plated on TSB regeneration plates containing kanamycin (100 μ/ml). Number of Km' transformants was caluculated by multiplying the dilution factor for each plate and indicated the number of Km' per ml.

mg/ml of the enzyme, whereas, above 370 ng/ml of plasmid DNA, the transformation efficiency with 2.5 mg/ml of lysozyme was slightly lower than that with 2.5 μg/ml of enzyme. These results indicate that this transformation system can be used for shotgun cloning experiments.

Comparison of transformation efficiency between protoplasts and competent cells

The results of transformation experiments were summarized in Table 1. The final DNA concentration in the transformation mixtures was 370 ng/ml. Competent cells transformation was carried out according to the method described previously (Aumayr et al., 1981). The transformation frequency of competent cells was 3.6 $x\,10^{-5}$ per 370 ng of plasmid DNA, and the number of Km' transformants was 4.0 x 10. The highest efficiency of protoplast transformation was performed with protoplasted cells with 2.5 μg/ml of lysozyme, and the number of Km' transformants reached 1.9X 10^4, which corresponds to 1.3 X 10^{-1} as frequency. Its efficiency was 10^3 fold higher than that of competent cells transformation. The transformation efficiency of *B.megaterium* protoplasts with pUB110 was $8.5x\ 10^4$ per μg of plasmid pUB110. This value was found to be considerably higher even at selective pressure of neomycin (100 μg/ml), compared with our obtained result (1.9 x 10" Km' transformants) under high selective pressure at 100 μg/ml of kanamycin. Recently, Takubo *et al.* (1990) reported that spore-spheroplast transformation of *B. megaterium* was developed and that the number of Km' transformants was 1.1 X 10^4, which corresponds to 1.1 X 10^{-5} as frequency.

In summary, it is quite important for successful protoplast transformation to achieve high regeneration ratio of protoplasted cells with lysozyme, and, consequently, to obtain efficient utilization of DNA in the transformation process.

Table 1 Transformation frequencies of protoplasts and competent cells

Transformation system	Lysozyme conc.	Percent of formation		Percent of regeneration[c]	Conc. of Km (μg/ml)	No. of Km'	Frequency of Km'
		round	TSB[b]				
Protoplasts	2.5μg/ml	25.1 %	92.0 %	100 %	100	1.9 x 10^4	1.3 $\times 10^{-1}$
	2.5mg/ml	99.9 %	99.9 %	20.8 %	100	1.4 X 10^3	1.0 $\times 10^{-2}$
Competent cells[d]					5	3.5 X 10^2	3.2 $\times 10^{-4}$
			–		25	1.5 X 10'	1.4 X 10^{-4}
					100	4.0 X 10	3.6 $\times 10^{-5}$

DNA used for protoplasts and competent cell transformation was 370 ng/ml of plasmid pUB110.
"The number of protoplasts formed was counted with microscopic observation.
"The number of protoplasts was estimated by counting the colonies on TSB and sodium succinate- and $MgCl_2$-free TSB agar plates.
'The number of regenerated cells was determined by plating the nontransformed protoplasts on regeneration medium without kanamycin. In this experiments, the number of total cells per ml was 1.5×10^7.
[d]The number of total cells per ml was 1.1 X 10^8.

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