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Rodprapakorn, Mangkorn

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

Doi, Katsumi

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

Ogata, Seiya

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

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## **Protoplast Regeneration and Transformation of Thiostrepton-Producing *Streptomyces Zaurentii* ATCC 31255**

Mangkorn Rodprapakorn, Katsumi Doi and Seiya Ogata\*

Microbial Genetics Division, Institute of Genetic Resources,  
Faculty of Agriculture, Kyushu University, Fukuoka 812-81, Japan  
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The optimum condition for the protoplast formation and regeneration of *Streptomyces laurentii* ATCC 31255 was studied. The effective formation and regeneration of protoplasts were obtained by using the mycelia grown to the stationary phase in the presence of 0.4% glycine, and by treatment with 1 mg/ml of lysozyme in PWP buffer. When plasmids, pMCP5 and its derivative pYK3 (smaller in size) having a kanamycin resistance gene, were transformed into the protoplasts by using 20% (V/V) PEG 2000 in PWP buffer, the high number of transformants were obtained at a frequency of  $4.3 \times 10^5$  and  $3.4 \times 10^4$  per  $\mu\text{g}$  DNA of pMCP5 and pYK3, respectively.

### INTRODUCTION

*Streptomyces* species produce many industrially important antibiotics and physiologically active substances. The protoplasts of these microorganisms are useful in genetic studies and molecular breedings using protoplast transformation or transfection and fusion techniques. The basic conditions for handling *Streptomyces* protoplasts were developed largely by Okanishi *et al.* (1974) and adapted with minor modifications by many investigators (Shirahama *et al.*, 1981; Pigac *et al.*, 1982 ; Ogawa *et al.*, 1983). It has been found that the frequency of protoplast formation and regeneration varies according to the species used (Bibb *et al.*, 1980 ; Ogawa *et al.*, 1983). Furthermore, as some *Streptomyces* species form only unstable protoplasts or do not form them at all, some modified procedures have been used (Okanishi *et al.*, 1974 ; Shirahama *et al.*, 1981; Baltz and Matsushima, 1981; Ogawa *et al.*, 1983 ; Ogata *et al.*, 1984 ; Yamashita *et al.*, 1985 ; Hranueli *et al.*, 1986 ; Yamamoto *et al.*, 1986 ; Anne *et al.*, 1990 ; Jandova and Tichy, 1990).

Thus, the first step of this work was to demonstrate the protoplast formation and regeneration in thiostrepton-producing *Streptomyces Zaurentii* ATCC 31255 (Trejo *et al.*, 1977) which had not been studied. The elucidation of the protoplast formation and regeneration procedures will help in genetic studies of *S. laurentii* involving fusion, transformation and transfection. In this paper, the conditions for the protoplast formation and regeneration of this strain are clearly described.

Recently some efficient transformation (Bibb *et al.*, 1978 ; Chater *et al.*, 1982 ; Feitelson & Hopwood, 1983 ; Nakano *et al.*, 1984) system for genus *Streptomyces* was established using their protoplasts. After succeeding in preparing the high frequency protoplast regeneration, the transformation ability of this protoplast was examined as

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\* Corresponding author.

the second step of this work. To check efficiency of protoplast transformation and compatibility between protoplasts of this strain and kanamycin resistance gene (*kmr*) carrying plasmid pMCP5 (Nakano *et al.*, 1984) and its derivative pYK3, these two plasmids were introduced into the obtained protoplast by polyethylene-glycol (PEG)-assisted transformation (Suarez and Chater, 1980).

## MATERIALS AND METHODS

### **Bacteria and plasmids**

*Streptomyces laurentii* ATCC 31255, plasmid pMCP5 (ca. 14 kb ; Nakano *et al.*, 1974) and its derivative pYK3 (ca. 8 kb) were used throughout this work. Plasmid pMCP5 was received from Prof. H. Ogawara of Meiji College of Pharmacy, and plasmid pYK3 was recently produced in Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University.

### **Media, buffer and reagents**

Four kinds of media were used for the cultivation, protoplast regeneration and transformation. Bennett medium and MG-1 medium (Ogata *et al.*, 1985) were used for the precultivation and main cultivation, respectively. For the protoplast regeneration and transformation, osmotically stabilized agar regeneration R2YE medium and Soft Nutrient Agar (SNA) medium (Hopwood *et al.*, 1985) were used. PWP buffer was prepared as described by Shirahama *et al.* (1981).

PEG 2000 was purchased from Nakarai Chemicals Ltd. Egg white lysozyme was from Seikagaku Kogyo Ltd.

### **Preparation and regeneration of protoplast, and estimation of regeneration frequency**

Spores (about  $10^8$  colony forming units, cfu) were inoculated into 50 ml of Bennett medium and cultivated for 24 hr at 28°C on a rotary shaker (250 rpm). This cultivated broth was transferred (1% v/v) to MG-1 medium, and cultivation was further carried out under the same conditions as above for 24 hr except for the study of the effect of cultivation time on protoplast formation and regeneration which were harvested at 12, 16, 20, 24 and 28 hrs. Then the mycelia were harvested by centrifugation (5,000 g), washed twice in 10.3% sucrose solution, suspended in PWP buffer containing with lysozyme (1 mg/ml), and incubated at 30°C, for 40-60 min with gentle shaking. After that the protoplast suspension was filtered twice through a sterilized cotton-filter to remove the intact mycelia and cell debris, and then the protoplasts in the filtrate were pelleted by centrifugation at 500 g for 10 min. The protoplasts precipitated were gently suspended in 1 ml of PWP buffer. The number of protoplasts converted was estimated by microscopic counts of 10 random haemocytometer fields. The percentage of regeneration was estimated by comparing the colony forming units on R2YE agar plates with the number of protoplasts present. The protoplast suspension was also diluted with water and plated on the same agar plate to determine the number of the osmotically resistant cells (nonprotoplast cells) in the protoplast suspension.

### Preparation of plasmid DNA

Plasmid pMCP5 DNA and pYK3 DNA were prepared by alkaline lysis method (Hopwood et al., 1985) from *S. Zividans 1326* and a derivative of *S. azureus* PK100C, respectively. Then the DNAs were purified by CsCl-EtBr density gradient centrifugation (Maniatis, 1982).

### Transformation of protoplast

The plasmids pMCP5 and pYK3, which carried kanamycin resistance gene as marker, were introduced into the protoplasts by polyethylene-glycol (PEG)-assisted transformation method. A 50 ml of protoplasts ( $2 \times 10^9$ ) was transferred to an Eppendorf tube. Subsequently, 1  $\mu$ g of plasmid DNAs in 50  $\mu$ l of PWP buffer, and 400  $\mu$ l of a sterilized solution of 20% (v/v) PEG 2000 in PWP buffer were added to the protoplast suspension. After carefully mixing, the suspension was incubated at 32°C for 3 min. These protoplasts were collected by centrifugation (600 g, 10 min) and gently resuspended in 100  $\mu$ l of PWP buffer. Then, serial dilution was performed in PWP buffer, and each of 50  $\mu$ l of diluted suspension was immediately spread on R2YE regenerating plates. Plates were pre-incubated at 28°C. To select transformants, the plates were overlayed with SNA medium (3 ml/plate) containing kanamycin (50  $\mu$ g/ml) after the 20 hr pre-incubation.

### Stability of plasmids in transformants

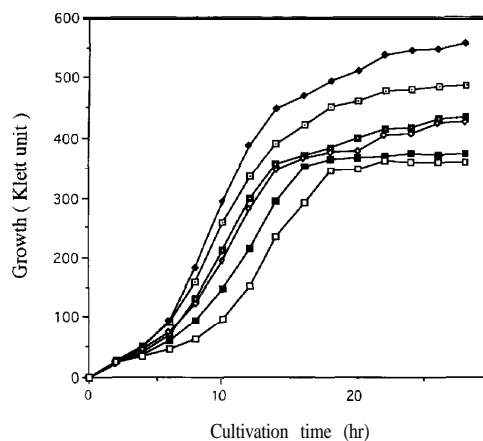
To check the stability of plasmids in the transformants, the colonies that grew on the R2YE agar plate were transplanted for several times onto a Bennett plate containing 50  $\mu$ g/ml of kanamycin.

## RESULTS

### Effect of concentration of glycine in MG-1 medium on protoplast formation and regeneration

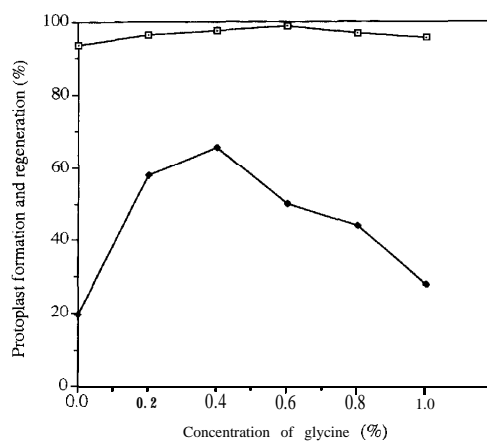
Okanishi et al. (1974) showed that the formation of *Streptomyces* protoplasts can be accomplished by using mycelia grown in the presence of sub-inhibitory concentration of glycine. They determined a suitable glycine concentration for the protoplast formation of certain *Streptomyces* species, but *S. Z Laurentii* had not been studied. In Fig. 1, increasing glycine concentration in range of 0–1% (w/v) reduced the growth of *S. Z Laurentii* except at 0.2% glycine which gave a slight higher growth rate than non-glycine-supplement.

As shown in Fig. 2, although the most effective protoplast formation was obtained from the mycelia cultured in 0.6% glycine, the highest regeneration frequency (65%) was obtained from the mycelium cultured in 0.4% glycine. Addition of glycine showed the more effective protoplast formation and regeneration than without glycine. However, the frequency of protoplast regeneration was decreased at the glycine concentration higher than 0.4%. Thus, glycine in this range (0–1%) promoted the protoplast formation and regeneration in *S. Z Laurentii* ATCC 31255.



**Fig. 1.** Effect of concentration of glycine in MG-1 medium on growth of *S. laurentii*.

—□—, 0% glycine ; —●—, 0.2% glycine ;  
 —◻—, 0.4% glycine ; —◊—, 0.6% glycine ;  
 —■—, 0.8% glycine; —□—, 1.0% glycine.



**Fig. 2.** Effect of concentration of glycine in MG-1 medium on protoplast formation and regeneration of *S. laurentii*.

—□—, protoplast formation;  
 —●—, protoplast regeneration.

### Effect of concentration of lysozyme on protoplast formation and regeneration

Effect of concentration of lysozyme on the protoplast formation and regeneration was examined in a range of 0.5-10 mg lysozyme/ml as shown in Fig. 3. The optimum concentration of lysozyme for protoplast formation and regeneration of this strain was 1 mg/ml. Higher concentration of lysozyme did not inhibit the protoplast formation, but resulted to a reduction in protoplast regeneration. It is possible that the excess

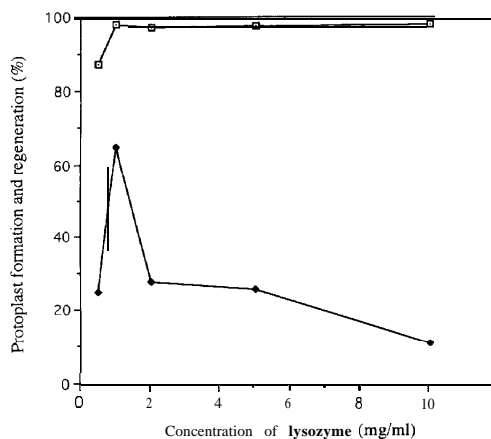


Fig. 3. Effect of concentration of lysozyme on protoplast formation and regeneration of *S. laurentii*.

□ , protoplast formation ;  
 —●—, protoplast regeneration.

lysozyme digests the newly formed cell walls or induces an aggregation of protoplasts.

#### Effect of cell growth phase on protoplast formation and regeneration

Baltz (1978) reported that the frequency of protoplast regeneration of *Streptomyces fradiae* and *Streptomyces griseofuscus* varied during the growth cycle and was highest when protoplasts were prepared from cells in the transition phase between the exponential and stationary phases. Furthermore, Garcia-Dominguez et al. (1987) obtained a high efficient rate of protoplast regeneration of *Streptomyces clavuligerus* by using mycelium which was harvested from the end of the exponential phase or stationary phase. In contrast, Okanishi et al. (1974) showed that protoplasts can be formed and regenerated efficiently from young mycelium at the early or middle exponential phase. The protoplast regeneration was greatly affected by the growth phase of the culture (Garcia-Dominguez et al., 1987).

To find the optimum growth phase of this strain for the protoplast formation and regeneration, the mycelium was harvested at the following phases of growth: early stage (12 hr), middle stage (16 hr), and end of exponential phase (20 hr), early stationary phase (24 hr) and lately stationary phase of growth (28 hr) (see Fig. 1). The mycelium harvested from early stationary phase (24 hr), gave the highest frequency of protoplast regeneration (ca. 65%), while all of growth phases showed quite similar efficiency of protoplast formation, as shown in Fig. 4.

#### Transformation of protoplasts

Plasmids pMCP5 and pYK3 DNAs were introduced into the protoplasts of *S. laurentii* by a PEG-assisted transformation method. Kanamycin resistant transformants were obtained at high frequency of  $4.3 \times 10^5$  and  $3.4 \times 10^4$  per  $\mu\text{g}$  DNA of pMCP5

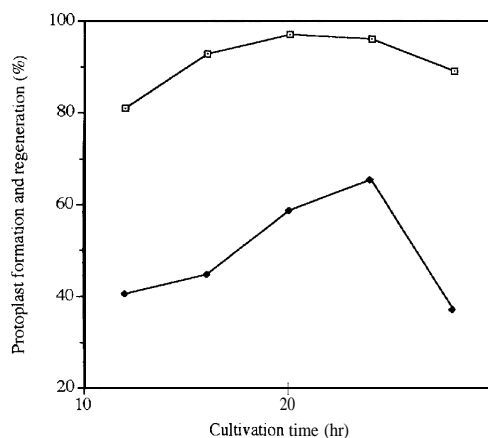


Fig. 4. Effect of cultivation time on protoplast formation and regeneration of *S. laurentii* in MG-1 medium.

—□—, protoplast formation ;  
—●—, protoplast regeneration.

Table 1. Frequency of transformation of *S. laurentii* ATCC 31255.

plasmid	transformants/ $\mu$ g DNA
pMCP5	$4.3 \times 10^5$
pYK3	$3.4 \times 10^4$

and pYK3, respectively, as shown in Table 1.

However, the stability test of plasmids pMCP5 and pYK3 in the transformants showed that they rapidly lost both plasmids. This instability of introduced plasmids may be due to the incompatibility between the exogenous plasmids pMCP5 or pYK3 and an endogenous giant plasmid pSLL (unpublished data).

## DISCUSSION

The protoplasts of *S. laurentii* ATCC 31255 could be easily prepared and could be regenerated at a high frequency under the optimum conditions such as : the cultivation in a MG-1 medium containing 0.4% glycine at 28°C for 24 hr ; and treatment with 1 mg lysozyme/ml in PWP buffer at 30°C for 40-60 min. The ease of protoplast formation in this strain may be due to its unique characteristic of the mycelium spontaneously becoming short fragments or rods of varying length in shaken culture (Trejo *et al.*, 1977). These fragments of mycelia simply change to the protoplasts by the treatment of egg-white lysozyme. On the other hand, for the protoplast preparation of many other streptomycetes, the large mycelia need to be fragmented by ultrasonic treatment to avoid accumulation of large mycelial masses before lysozyme treatment (Baltz,

1978).

Presence of a giant plasmid pSLL in *S. laurentii* ATCC 31255 give very significant effect on the stability of incorporated foreign DNAs such as plasmids pMCP5 and pYK3. The incorporated plasmids would be eliminated during the mycelial growth due to the incompatibility between the resident plasmid pSLL and exogenous plasmids. To overcome this incompatibility, we are trying to find out the pSLL-cured derivatives of *S. laurentii* and developing them to be a useful host.

### ACKNOWLEDGEMENT

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