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Protoplast Fusion of *Streptomyces tubercidicus*

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*Streptomyces tubercidicus*의 원형질체 융합

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Abstract: A procedure for the preparation, regeneration and fusion of protoplasts of *Streptomyces tubercidicus* was confirmed. Also, protoplast releasing processes from mycelia were observed by scanning electron microscope. Three types of protoplasts releasing processes-from the hyphal tip, hyphal end regions and lateral regions of the hyphae-were observed. More than 17% regeneration efficiency was obtained by regeneration medium that is composed of tryptone-yeast extract -sodium acetate-$MgCl_2$-$CaCl_2$-sucrose. Optimal concentrations of Ca^{++}, Mg^{++} and sucrose in the regeneration medium were 50 mM, 5 mM, 0.4-0.5 M respectively. Above 30% of fusion frequency of the protoplasts derived from two auxotrophic strains of *S. tubercidicus* was induced by polyethylene glycol 4000 (60% w/v).
Key words: *Streptomyces tubercidicus*, protoplast preparation, regeneration, fusion, protoplast releasing process.

The prokaryotic genus *Streptomyces* is curious if not phenomenal in its ability to synthesize antibiotics of diverse chemical structure and mode of action (Hopwood and Merrick, 1977, Hopowood, D.A., 1967). Of the 3000 or so antibiotics discovered through about 1974, about 70% are produced by *Streptomyces* (Hopwood and Merrick, 1977). The most significant advances in *Streptomyces* genetics involve protoplast fusion, facilitating the high frequency of *in vivo* genetic recombination and the transformation or transfection of protoplasts by plasmid or actinophage DNA, resulting in the development of *in vitro* recombinant DNA technology (Hopwood and Chater, 1980). Therefore, procedures for protoplast preparation and regeneration of different species and even of different strains are indispensible.

The basic conditions for handling *Streptomyces* protoplast were largely developed by Okanish *et al.* (1974) and adapted with minor modifications by many investigators (Hopwood, 1981, Sagara *et al.*, 1971, Shirihama *et al.*, 1981).

In this paper, we describe procedure for the preparation, regeneration and fusion of protoplasts of tubercidin producing *Streptomyces tubercidicus* and report morphological details of the protoplast releasing process itself.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were *Streptomyces tubercidicus* ATCC 25502, *S. tuber-*

cidicus 412(his) and *S. tubercidicus* 115(ade). *S. tubercidicus* 412 and 115 were auxotrophic mutants obtained by u.v. mutagenesis from *S. tubercidicus* ATCC 25502 in our laboratory.

Growth of mycelia and protoplast preparation

The strains were cultivated in medium containing tryptic soy broth (30g/*l*), sucrose (0.5 M), glycine 2% (w/v). The mycelia were harvested by centrifugation and were washed with a solution of 0.5 M sucrose. For the preparation of *S. tubercidicus* protoplasts, the following procedure was used. Washed mycelia were incubated in P buffer (Okanish *et al.*, 1974) containing 0.5 M sucrose with the addition of 2 mg of lysozyme(Sigma) per m*l* at 29℃ for 90 min. And then, protoplasts were filtered by cotton wool and washed by P buffer three times. The number of protoplasts was determined with a hemacytometer.

Reversion of protoplasts

Purified protoplasts were diluted in P buffer and plated on regeneration media for viable counts to determine protoplasts reversion. The plates were incubated at 29℃ for more than 3 weeks. Growing colonies were counted, and the reversion rate expressed as a percentage of the total number of inoculated protoplasts. Regeneration of protoplasts was monitored on following regeneration medium containing tryptone (5g/*l*), yeast extract, (0.5g/*l*), sodium acetate (0.2g/*l*), sucrose (0.5M), $MgCl_2 \cdot 6H_2O$ (5mM), $CaCl_2$ (50mM). Colony formation from nonprotoplasted unit was determined by diluting protoplasts in water and plating on nonhypertonic regeneration medium.

Fusion of protoplasts

After suspending in P buffer, protoplast (about 1×10^9 of each auxotrophic strain 412 and 115) were mixed and sedimented by centrifugation to 1500×g for 15 min. The pelleted protoplasts were resuspended gently with P buffer and prewarmed (29℃) solution of 60% (w/v) PEG 4000 in P buffer was added. After incubaction at 29℃ for 5 min serial dilution (0.1m*l* amount) of fused protoplasts in P buffer were plated on regeneration media and incubated at 29℃ until no new colonies were formed. Each colony on regeneration media was transferred to complete medium (Bennet's agar) and minimal medium (Hopwood *et al.*, 1985) with toothpick. Fusion frequency was given as the ratio of prototrophic colonies growing on MM to the total number of viable colonies on CM expressed as a percentage.

Electron microscopy

Normal mycelia and lysozyme treated mycelia were harvested by centrifugation and observed with scanning electron microscope (Hitachi S-450) as described by Hah *et al.* (1986).

RESULTS AND DISCUSSION

Optimal conditions for protoplast formation and regeneration

The effect of the growth phase on protoplast formation of *S. tubercidicus* ATCC 25502 is presented in Fig 1.

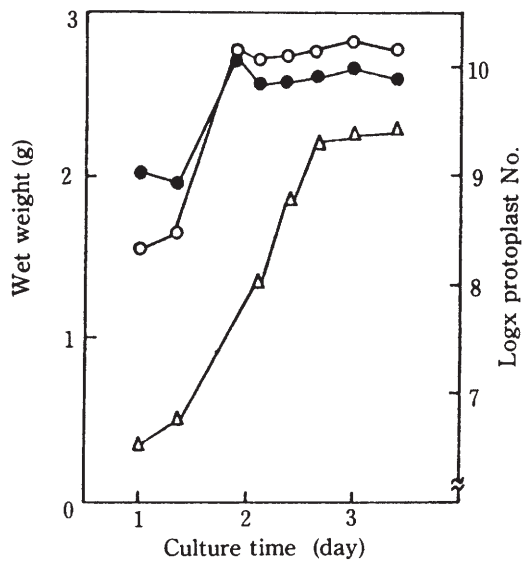

Fig.1. *Effect of growth phase on protoplast formation of S. tubercidicus ATCC 25502.*
○—○ : totol protoplast
●—● : total protoplast / wet weight mycelium(g)
△—△ : growth curve

Within 36h, few protoplasts were formed even with prolonged lysozyme treatment. After that time protoplasts could be readily obtained with large number. At transition stage between exponential and stationary growth, maximal total protoplasts and the best protoplast yield (No. of total protoplasts per mycelium wet weight(g)) were obtained. The effect of the growth phase on protoplast regeneration is presented in Fig 2. Same as the protoplast formation, protoplast regeneration was the best at critical transition stage between exponential and stationary growth. These results are similar with *S. fradiae* and *S. griseofus* (Baltz, 1978) and *S. rimousus*(Pigac *et al.*, 1982). Fig 1 and Fig 2 showed that 60 h culture was optimal for the protoplast preparation and regeneration of *S. tubercidicus* ATCC 25502. Regeneration frequency was about 17% at this growth stage. Protoplasts formation and regeneration are affected by osmotic stabilizers in *Streptomyces* (Okanishi *et al.*, 1974). To examine the effect of osmotic stabilizer on regeneration, protoplasts from *S. tubercidicus* was tested in

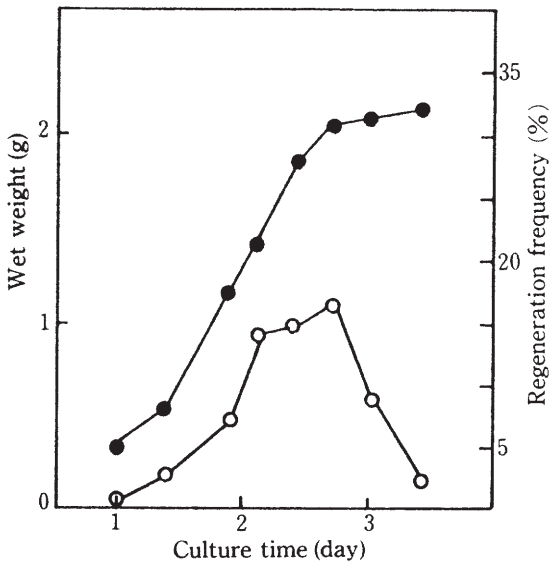

Fig.2. *Effect of growth phase on protoplast regeneration of S. tubercidicus ATCC 25502.*

○—○ : regeneration frequency
●—● : growth curve

Table 1. *Effect of Mgnn and Cann concentration on regeneration of S. tubercidicus* protoplasts.

$CaCl_2$ (mM)	$MgCl_2$ (mM)	protoplasts /ml	*CFU/ ml	regeneration frequency (%)
60	2	2.0×10^7	1.04×10^6	5.2
50	5	2.0×10^7	2.80×10^6	14.0
30	30	2.0×10^7	1.06×10^6	5.3
5	50	2.0×10^7	2.20×10^5	1.1
2	60	2.0×10^7	0	0
2	2	2.0×10^7	6.00×10^4	0.3

*CFU: Colony forming unit

a regeneration medium containing different concentrations of Ca^{++}, Mg^{++} and sucrose. It showed that 50 mM Ca^{++} and 5 mM Mg^{++} was optimal for regeneration efficiency on regeneration medium containing 0.5 M sucrose (Table 1), but regeneration efficiency are decreased as Mg^{++} concentration increase. Above result showed that high Ca^{++} concentration and low Mg^{++} concentration are essential for good regeneration of *S. tubercidicus* ATCC 25502. There is no regeneration on of protoplasts media containing 50 mM Ca^{++}, 5

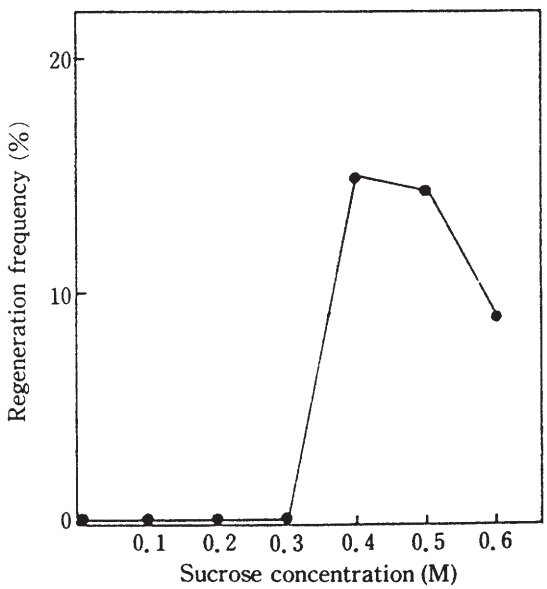

Fig.3. *Effect of sucrose concentration on protoplast regeneration of S. tubercidicus ATCC 25502.*

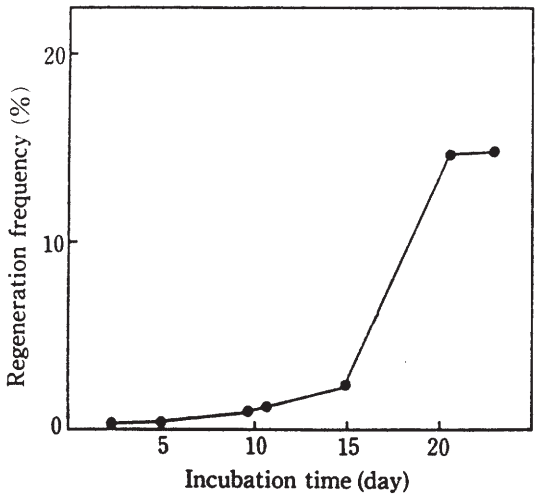

Fig.4. *Effect of incubation time on regeneration of S. tubercidicus ATCC 25502 at 29℃.*

mM Mg^{++} and below 0.3 M sucrose (Fig 3). Regeneration efficiency was optimal at 0.4 M -0.5 M sucrose and decreased at higher concentration (Fig 3). Table 1 and Fig 3 showed that regeneration of *S. tubercidicus* protoplast was very sensitive to the concentration of osmotic stabilizer. Protoplasts of *S. tubercidicus* was regenerated very slowly and more than 3 weeks was required for complete regeneration (Fig 4). However, in case of many other *Streptomyces* species 3-10 days are required for regeneration (Hopwood and Wright, 1978, Pigac *et al.*, 1982, Ochi *et al.*, 1979). It was observed that colonies from nonprotoplasted units appeared before those originating from regenerated protoplasts (data not shown). After complete protoplast regeneration, the fraction of nonprotoplasted units represented 0.01-0.05% of the entire population. However, the autoinhibition of protoplast regeneration by non-protoplasted unit (Baltz, 1978, Hopwood *et al.*, 1977) was not observed.

Morphological studies of protoplast releasing process

Normal mycelia of *S. tubercidicus* grew with many branches (Plate 1-a). It was observed that the protoplasts were formed at the hyphal tip by treatment with lysozyme solution

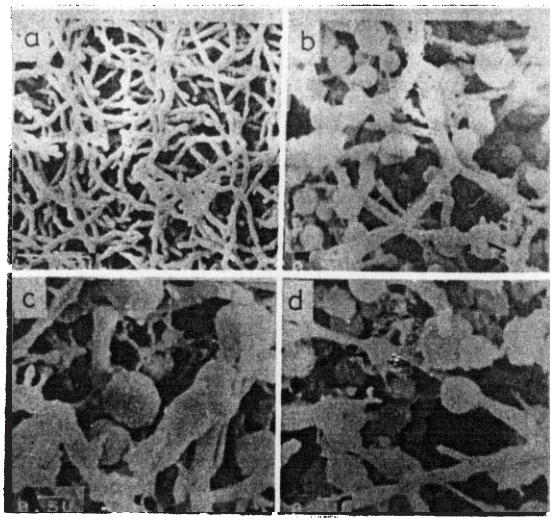

Plate 1. *Scanning electron microscopic observation on normal mycelium and protoplast releasing process of S. tubercidicus ATCC 25502.*

a: normal mycelium, b: protoplast released from hyphal tip, c: protoplast released from hyphal end regions, d: protoplast released from lateral regions of the hyphae

(Plate 1-b). The size of protoplasts produced from hyphal tip was 0.5 μm (mean value) and progressively increased. It was also observed that protoplasts were formed sequentially from

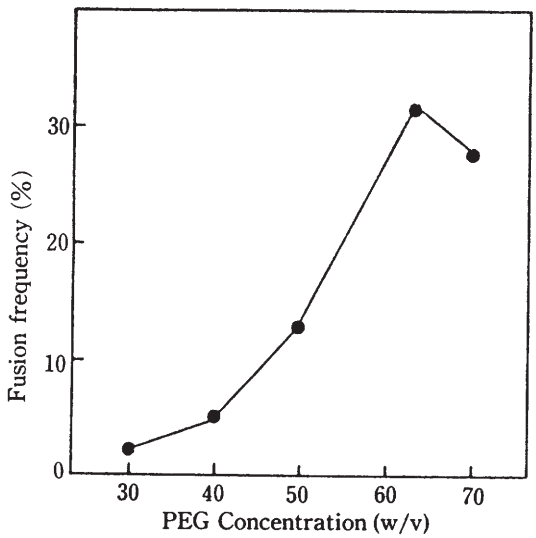

Fig.5. *Effect of PEG 4000 cocentration (w/v) on fusion frequency of S. tubercidicus 412 (his) and 115 (ade).*

hyphal end regions and lateral regions of the mycelium which was puffed entirely (Plate 1-c,d). In protoplast formation of *S. tubercidicus*, it was not found that protoplasts were produced by the fragmentation of mycelium in *Micromonospora rosaria* or other *Streptomyces* spp. (Kim, 1983).

Protoplast fusion

Fig 5 reveals the effects of the concentration of PEG 4000 on the frequency of prototrophic colony formation from fusion of protoplasts of *S. tubercidicus* 412 and 115. It showed that with increasing PEG 4000 concentration the fusion frequency increased, but above 60% (w/v) the fusion frequency declined a little. At 60% (w/v), high frequency of recombination (up to 33%) were obtained by the fusogenic procedure.

적 요

*Streptomyces tuberaidicus*의 원형질체 생성, 생성된 원형질체의 정상 균사체로의 환원 그리고 원형질체 융합에 대하여 조사하였다. 또한 균사체로부터 원형질체가 생성되는 과정을 주사전자현미경을 사용하여 관찰하였다. 원형질체는 균사의 끝부위에서 뿐만 아니라 균사 말단의 부풀어오른 부위 그리고 균사의 중간 부위에서도 생성이되는 세 종류의 원형질체 생성양상을 보였다. 원형질체의 정상균사체로의 환원률은 tryptone-yeast extract-sodium acctate -$MgCl_2$-$CaCl_2$-sucrose로 조성된 배지에서 대략 17%이었으며, 이때 Ca^{++}, Mg^{++} soccrose의 농도가 각각 50mM, 5mM, 0.4~0.5M일때 최적의 환원률을 보였다. *S. tuberaidicus*의 histidine과 adenine을 요구하는 두 균주의 원형질체간의 융합에서 30% 이상의 융합빈도를 얻을 수 있었다.

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