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Genetic Transformation of *Streptomyces caespitosus*

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Genetic transformation of *Streptomyces caespitosus* by plasmid pIJ 702 was carried out. Optimal conditions for the protoplast preparation of *Streptomyces caespitosus*, its regeneration, and its transformation by pIJ 702 were evaluated. Addition of 2% glycine to the culture broth was optimal for protoplast yield. Formation and regeneration of protoplasts were most efficient when the mycelium were harvested at between late log and stationary growth phase. The regeneration frequency of the protoplasts was 15% when the protoplasts were regenerated on R2YE agar media containing 0.5M sucrose. Under the best condition for protoplats regeneration, the optimal transformation frequency was achieved with 40% polyethylene glycol (M.W. 4,000) treatment for 2 minutes.

Key words: *Streptomyces caespitosus*, Protoplast, Regeneration, Transformation

INTRODUCTION

The genus Streptomyces contains many species which are industrially important because of the secondary metabolites they produce. Approximately 70% of the known antibiotics are produced by members of the genus *Streptomyces* (Epp *et al.*, 1988).

Although most of these antibiotics are well characterized chemically, only limited information is available on the biochemistry of antibiotic biosynthesis and on the genetics of the antibiotic-producing organisms (Ghisalba *et al.*, 1981). However , due to the recent development of cloning vectors and methods to introduce DNA into *Streptomyces* species and the early finding that antibiotic biosynthetic genes are usually clustered and adjoin the gene conferring resistance to the antibiotic produced, biosynthetic and resistance genes for different structral classes of antibiotics have been isolated and characterized (Katz *et al.*, 1983; Epp *et al.*, 1988).

Streptomyces caespitotus produces mitomycin C, an antibiotic belonging to the quinone group which contain a biosynthetically unique moiety, called an mC_7N unit, consisting of a six membered carbocyclic ring carrying an extra carbon and a nitrogen in a meta arrangement (Hata *et al.*, 1956; Horneman *et al.*, 1974; Anderson *et al.*, 1980). Extensive tracer and genetic experiments have demonstrated the shikimate pathway origin of this mC_7N unit, and have identified 3-amino-5-hydroxybenzoic acid (AHBA) as its proximate precursor (Rinehart Jr. *et al.*, 1982; Kim *et al.*, 1992). Also, antibiotics belonging to ansamycin group which include many commercially important antibiotics such as rifamcin B, contain an mC_7N unit, synthesized from a common aromatic precursor 3-amino-5-hydoxybenzoic acid deriving from the shikimate pathway (Ghisalba *et al.*, 1981).

We are trying to clone the mitomycin biosynthetic genes from *S. caespitotus* in order to study the biochemistry and genetics of the mitomycin biosynthesis. As the first step, the optimal conditions for protoplast formation, the subsequent regeneration to normal mycelia and protoplast tansformation system of *S. caespitotus* were examined.

MATERIALS AND METHODS

Microorganisms, plasmid, and culture conditions

Streptomyces caespitosus KCTC 9096 and plasmid pIJ 702 were used as a host strain and a vector DNA for transformation, respectively. *Streptomyces lividans* KCTC 1167 was used for isolation of plasmid pIJ 702 carrying the thiostrepton resistance gene(*tsr*) and the tyrosinase gene(*mel*) (Hopwood *et al.*, 1985). *S. caespitosus* was grown on oatmeal-yeast extract-malt extract (OMYM) medium (Nakamura *et al.*, 1988) at 27℃ for 10 days and resulting spores were used for inoculation

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Table I. Effects of glycine on the growth* and protoplast formation of *Streptomyces caespitosus*

Glycine concentration(%)	0	0.5	1	1.5	2	3	4
Dry cell weight(DCW, mg)	60	42	21	13	12	10	9
Protoplast yield(No./mg DCW)	2×10^4	7×10^6	2×10^8	8×10^8	1.3×10^9	1.5×10^9	1.6×10^9

**Streptomyces caespitosus* was grown in YEME liquid medium containing glycine and harvested after 36 hour cultivation.

of various culture media.

Production of protoplasts

S. caespitosus was cultivated in yeast extract-malt extract (YEME) liquid medium (Hopwood *et al.*, 1985) containing 0-4% glycine. The cells were harvested by centrifugation, washed twice with 0.3 M sucrose solution and then suspended in protoplast (P) buffer (Okanishi *et al.*, 1974). Lysozyme dissolved in P buffer was filter-sterilized and added to the final concentrations of 2 mg/ml. The resulting mixture was incubated at 30℃ for 60 minutes and filtered by cotton wool and washed by P buffer three times. The number of protoplasts was determined with a hemacytometer.

Regeneration of protoplasts

Purified protoplasts obtained by the above procedure were diluted in P buffer and plated on various regeneration media. The number of colonies was determined after 10 days of incubation. Colony formation from nonprotoplasted unit was determined by diluting protoplasts in distilled water and plating on nonhypertonic regeneration medium. Regeneration rate was expressed as a percentage of regenerants per total CFU (colony forming unit).

Preparation of plasmid pIJ702

Plasmid pIJ 702 was isolated from mycelia of *S. lividans* KCTC 1167 grown in YEME broth containing thiostrepton (20 μg/ml) by an alkaline-SDS method as described by Kieser (1984). Agarose gel electrophoresis of DNA was performed as described by Hopwood *et al.* (1985).

Transformation

The protoplasts(5×10^8) of *S. caespitosus* were suspended in a small volume of P buffer and mixed with the plasmid pIJ 702 (100 ng) in 20 μl of TE (Tris-EDTA) buffer. Transformation was done by the addition of 0.5 ml of polyethylene glycol 4,000 dissolved in T (transformation) medium as described previously by Hopwood *et al.* (1985).

For direct selection of antibiotic resistant transformants, protoplasts were plated on R_2YE agar media (Hopwood *et al.*, 1985) and incubated for 15-18 hours at 27℃ to allow for expression of the resistance genes and then the plates were overlaid with a nutrient soft agar containing thiostrepton to give a final concentration of 40 μg/ml. Thiostrepton resistant *S. caespitosus* transformants were counted after 7-10 days of the incubation.

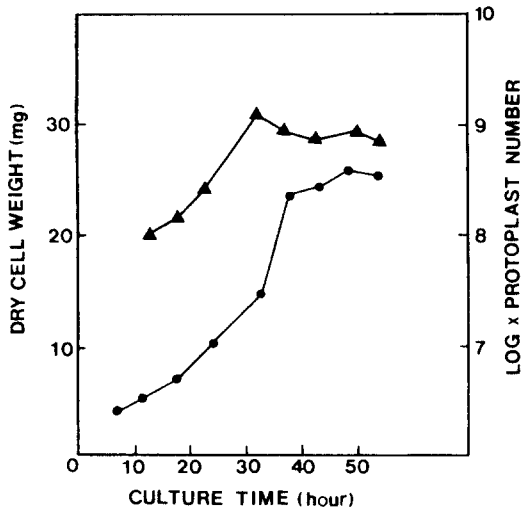

Fig. 1. Effect of growth phase on the protoplast formation of *S. caespitosus* KCTC 9096.
●: Growth curve, ▲: Protoplast/Dry cell weight (mg)

RESULTS AND DISCUSSION

Protoplast formation and regeneration

To determine the optimal glycine concentration for protoplast formation, a serious cultures containing 0-4% (w/v) glycine were cultivated for 36 hours, harvested, and subjected to the lysozyme. As shown in Table I, addition of 2% glycine to the culture broth was optimal considering cell growth and protoplast yield. There have been many reports correlating mycelial growth phase with the efficiency of protoplast preparation and regeneration. Baltz (1978) reported a critical transition stage between exponential and stationary growth for the preparation and regeneration of *S. fradiae* and *S. griseofuscus* protoplasts. Similiar results were reported for *S. rimosus* (Pigac *et al.*, 1982), *S. tubercidicus* (Yoo *et al.*, 1986) and *S. lividans* (Hopwood, 1981). The effect of the growth phase on protoplast formation of *S. caespitosus* is presented in Fig. 1. The protoplast yield was the highest when the my-

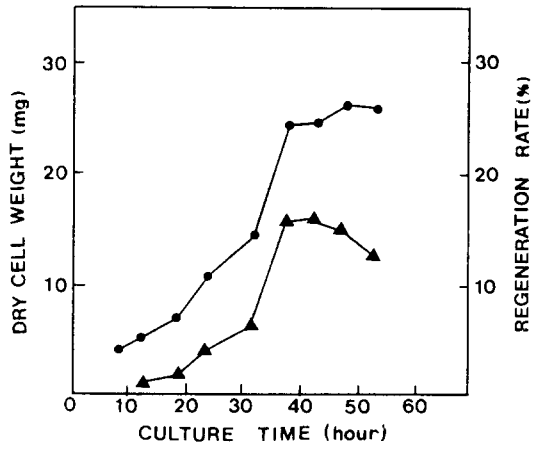

Fig. 2. Effect of growth phase on the protoplast regeneration of *S. caespitosus* KCTC 9096.
●: Dry cell weight (mg), ▲: Regeneration rate (%)

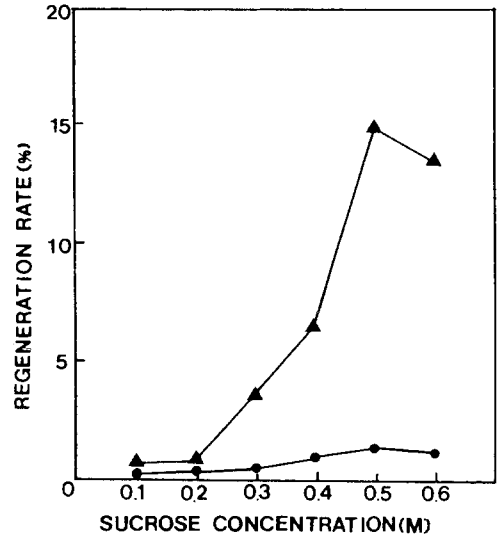

Fig. 3. Effect of surcrose concentration on the protoplast regeneration of *S. caespitosus* KCTC 9096.
●: Rc medium, ▲: R_2YE medium

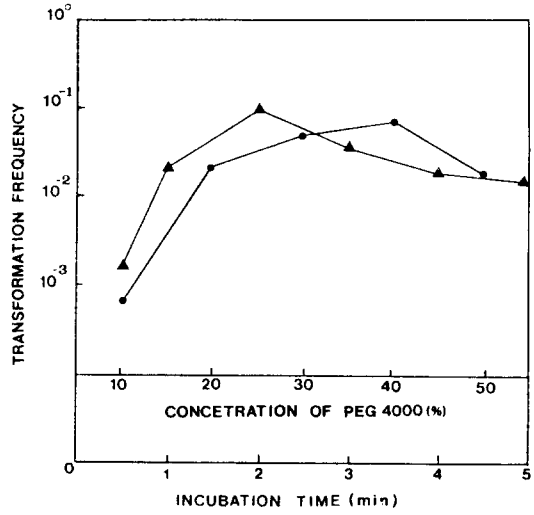

Fig. 4. Effect of PEG 4000 concentration and incubation time on the transformation of *S. caespitosus* KCTC 9096 by pIJ 702.
●: Conc. of PEG 4000 (%), ▲: Incubation time (min)

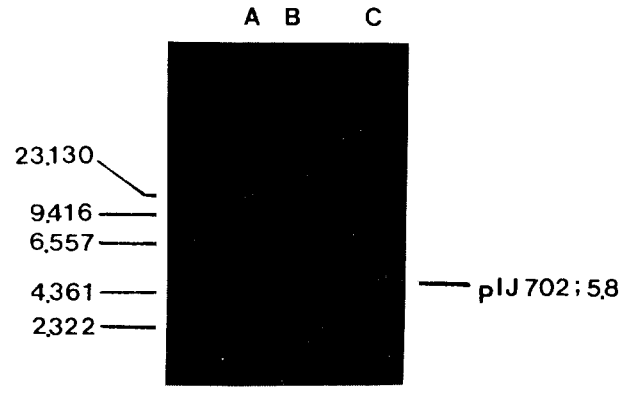

Fig. 5. Electrophoretic pattern of pIJ 702 obtained from *S. caespitosus* and *S. lividans* KCTC 1167.
A: Size marker; λ/HindIII, B: Transformant of *S. caespitosus* by pIJ 702, C: *S. lividans* KCTC 1167 containing pIJ 702

celia at transition stage between mid-exponential and stationnary growth were used. Also, protoplasts prepared from mycelia taken from transition stage between late-exponential and stationary growth was required for optimal regeneration (Fig. 2). Therefore, it was possible to prepare protoplasts that were capable of good regeneration for use in transformation.

Protoplasts regeneration was affected by media composition and osmotic stabilizer concentration (Okanishi *et al.*, 1974; Shirahama *et al.*, 1981). To examine the optimal regeneration media and effect of the osmotic stabilizer concentration on regeneration, protoplasts prepared from *S. caespitosus* were tested in Rc (complete regeneration) medium (Yoo *et al.*, 1986) and R_2YE medium containing various concentrations of sucrose. As shown in Fig. 3, R_2YE media is more efficient than Rc media, and optimal concentration of sucrose was observed to be 0.5 M, and the protoplast regeneration frequency dropped when the sucrose concentration wae lower than 0.5 M. The regeneration frequency was about 15% under the best condition for protoplats regeneration.

Resistance of *S. caespitosus* and *S. lividans* against antibiotics

To determine the resistance of *S. caespitosus* and *S. lividans* for thiostrepton and mitomycin C, *S. lividans* and *S. caespitosus* were cultivated on R_2YE agar medium containing various concentrations of thiostrepton and mitomycin C. As shown in Table II, *S. caespitosus*

Table II. Effect of thiostrepton and mitomycin C on the growth of *Streptomyces lividans* and *Streptomyces caespitosus*

Strains	Thiostrepton (μg/ml)					Mitomycin-C (μg/ml)		
	12.5	25	50	100	500	10	50	200
S. lividans KCTC 1176 (plasmd pIJ 702)	+	+	+	+	+	+	−	−
S. lividans 1326	+	−	−	−	−	+	−	−
S. caespitosus	−	−	−	−	−	+	+	%

S. livdans and *S. caespitosus* were cultivated on R_2YE agar medium containing various concentrations of thiostrepton and mitomycin C. +: growth, −: no growth.

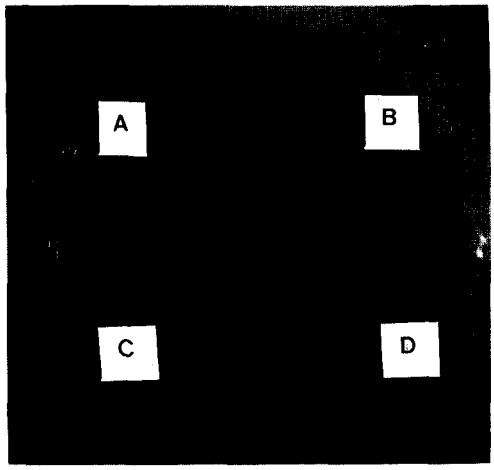

Fig. 6. Melanin production of *S. caespitosus* transformants on pepton-yeast extract iron agar medium.
A: *S. caespitosus* KCTC 9096, B: Transformant of *S. caespitosus* by pIJ 702, C: *S. lividans* KCTC 1326, D: *S. lividans* KCTC 1167 containing pIJ 702

was very sensitive to thiostrepton and resistant to mitomycin which is its own product up to 200 μg/ml. So, it appears that thiostrepton resistance is a good marker for selection of transformants of *S. caespitosus* which are transformed by plasmid pIJ 702. *S. lividasns* harvouring pIJ702 was very resistant to thiostrepton, but *S. lividans* does not have pIJ 702 was very sensitive (Table II). Both strains of *S. lividans* were sensitive to mitomycin C. These results suggested that *S. lividans* can be used as a host for cloning the genes for mitomycin C biosynthesis and resistance.

Transformation of protoplast

Polyethylene glycol induces plasmid transformation of *Streptomyces* protoplasts (Matsushima and Baltz, 1985). In the *S. lividans* (Hopwood *et al.*, 1985), 20% PEG induced maximum transformation. In our studies on transformation of *S. caespitosus*, we found that 30-40% PEG were most efficient (Fig. 4). Lower transformation frequencies were obtained when below 20% PEG were used. To determine the time required for optimal transformation, PEG treated transformation mixture was incubated for various times at room temperature before plating on regeneration media. Effective transformation frequency (10%) was obtained from the reaction mixture incubated for 2 minutes, incubation for longer time decreased the transformation frequencies (Fig. 4).

Plasmid obtained from transformants showed the same band pattern as that of pIJ 702 on agarose gel electrophoresis (Fig. 5). Also, melanine production of transformants on peptone-yeast extract iron agar and tyrosine agar medium (Shirling and Gottlieb, 1966) confirmed that transformants of *S. caespitosus* have the plasmid pIJ 702 carrying the tyrosinase gene(*mel*) (Fig. 6).

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