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Cloning and Expression of the Extracellular β-Lactamase Gene from *Streptomyces* sp. SMF13 in *Streptomyces lividans*

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Cloning of the gene encoding extracellular β-lactamase from *Streptomyces* sp. SMF13 in a plasmid pIJ702 and expression of the gene in *Streptomyces lividans* were carried out. Optimal conditions for the formation of protoplasts of *S. lividans* and the regeneration of the protoplasts were evaluated. *Streptomyces* sp. SMF-13 was selected as a donor strain of β-lactamase gene and total DNA of the strain was partially digested with Sau3A I. DNA fragments ranged from 4 kb to 10 kb were ligated to pIJ702 at Bgl II site and then the ligated DNAs were transformed to the protoplasts of *S. lividans*. The transformation efficiency was $2\times10^3/\mu$g DNA for the ligated DNA mixture. One colony among a thousand colonies regenerated showed extracellular β-lactamase and the size of the inserted DNA fragment was estimated to be 3.94 kb. The β-lactamase activity in the culture broth of the recombinant strain was maximum at 3 days culture to be 1.0 unit/ml.

KEY WORDS □ ***Streptomyces lividans*, β-lactamase, cloning, protoplasts, β-lactam antibiotics, β-lactamase gene**

β-Lactam antibiotics are hydrolyzed to inactive forms of the corresponding compounds by β-lactamase. Although the β-lactamase was identified before the discovery of β-lactam antibiotics, various β-lactamases having different properties were identified after the wide applications of the antibiotics (1, 10). Pathogenic and various bacteria resistant to the β-lactam antibiotics were frequently isolated from the places where the antibiotics were widely disposed. The results indicated that the resistants acquired the ability to produce the β-lactamase through horizontal gene transferring routes (12). β-Lactamases were classified into four groups according to the substrate specificity and inhibitor profile (1). It was reported that *Streptomyces* spp. produced the β-lactam antibiotics as well as the β-lactamases (15). The β-lactamases produced in *Streptomyces* spp. were characterized to be the class 1 which were encoded by chromosomal DNA (3). In addition *Streptomyces* spp. produced proteinaceous β-lactamase inhibitor (5, 8, 19). However the molecular mechanisms regulating the production of these compounds remain to be understood. In this concept, we were trying to clone the genes encoding the proteins and to express in *Streptomyces lividans* in order to elucidate the regulatory mechanism. As the first step, the β-lactamase gene was cloned and expressed in *Streptomyces lividans*.

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MATERIALS AND METHODS

Microorganims, plasmids, and culture conditions

Streptomyces sp. SMF13 was used as a donor strain of genomic DNA for β-lactamase (7, 13). Plasmid pIJ702 and *S. lividans* 1326JI were used as a cloning vector and a host strain, respectively. Fig. 1 shows the restriction map of plasmid pIJ702 carrying the thiostrepton resistance gene (*tsr*) and the tyrosinase gene (*mel*) which were used as selection markers (9). The microorganisms were grown in a rich liquid medium (YEME) at 30°C using baffled flasks in an orbital shaking incubator (200 RPM). In protoplast preparation, cells were grown in the YEME medium containing 0.3 % of glycine. Another rich agar medium (R2YE) was used for the regeneration of protoplasts (16, 11). For the selection of recombinant harbouring foreign DNA, 20 μg/ml of thiostrepton was added to the R2YE medium . .

DNA manipulation, transformation, and screening of recombinants

Isolation and manipulation of DNA were carried out with the methods as described in elsewhere (6). Cells harvested from the late

exponential growth phase of the batch culture with glycine were treated with lysozyme (1 mg/ml as final concentration) for 15 min in TE buffer where 10.3% sucrose was added as an osmotic stabilizer. The more detail procedures for the protoplast formation, transformation, and regeneration of the transformed cells were followed with the methods (2). Since the plasmid pIJ702 (Fig. 1) carried a thiostrepton resistance gene (*tsr*), colonies developed on the medium containing thiostrepton were selected as the transformants harbouring either the vector plasmid or recombinant plasmid DNA. And further screening of the transformants harbouring recombinant plasmids was made to select the white colonies, because the cloning site of the plasmid pIJ702 was tyrosinase gene (*mel*) which was inactivated by the insertion. In order to make the final screening of recombinant harbouring β-lactamase gene, 4 ml of nitrocefin solution (250 μg/ml) was overlayed to the colonies regenerated on R2YE agar media containing 20 μg/ml of thiostrepton. Colonies forming red hallow zone around them were selected as recombinants producing β-lactamase by which nitrocefin was hydrolyzed to develop red color (3).

Analytical methods and chemicals

β-Lactamase activity was determined with the method of iodometric assay (17). One unit of β-lactamase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of penicillin G per min at 37°C. Thiostrepton was kindly donated from the Eli-Lilly and nitrocefin was provided by the Glaxo Group Ltd.

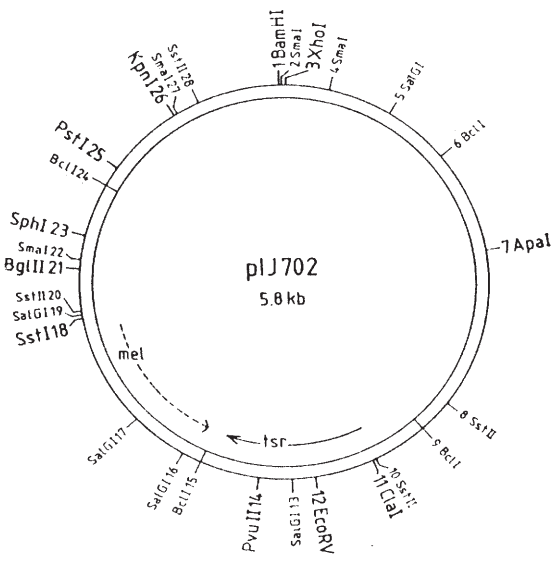

Fig. 1. *Restriction map of pIJ702 (9).*

RESULTS AND DISCUSSION

Selection of strains for cloning of β-lactamase gene

In order to select strains for cloning host and β-lactamase gene donor, extracellular activity of β-lactamase in culture broth of various strains of *Streptomyces* spp. was tested. As shown in Table 1, *Streptomyces* sp. SMF13 isolated from soil showed very high activity of β-lactamase, on the other hand, *S. lividans* 1326JI showed no activity of β-lactamase in the culture conditions. Hence, *Streptomyces* sp. SMF-13 was selected as a donor strain of β-lactamase and *S. lividans* 1326JI was used as a host strain for the expression of the cloned β-lactamase.

Table 1. *Production of β-lactamase in strains of Streptomyces* spp.*

Strain used	β-lactamase activity (U ml^{-1})
Streptomyces lividans 1326JI	0.00
Streptomyces sp. SMF-13	1.18
Streptomyces sp. SMF-209	0.07
Streptomyces sp. SMF-301	0.46
Streptomyces sp. SMF-313	0.07

*They were cultured in YEME medium at 30°C for 3 days.

Protoplast formation, regeneration, transformation, and screening

Cells grown in the rich medium YEME were very much branched and filamentous mycelia which were very tightly aggregated (Fig. 2A). However, the cells were converted to straight and readily dispersed by the addition of 0.3% of glycine to the YEME medium from the beginning of a batch culture (Fig. 2B) and very uniform protoplasts could be obtained after by the treatment of lysozyme (Fig. 2C). The efficiency of protoplast formation and that of regeneration from the protoplasts were estimated to be 98% and 13%, respectively. Chromosomal DNA (about 10^4 Kb) of the donor strain (SMF13) was isolated and digested with Sau3A I (0.13 unit/μg DNA) at 37°C for 1 h. DNA fragments ranged from 4 Kb to 10 Kb could be obtained by sucrose density gradient (10-40%) centrifugation. The DNA fragments were ligated to the plasmid pIJ702 DNA which was digested with Bgl II and dephosphorylated by calf intestinal phosphatase (the mass ratio between chromosomal DNA and plasmid DNA was 5/1). As shown in Fig. 3, the plasmid band (5.6 Kb) was detected clearly in the mixture before ligation (lane 2 in Fig. 3), while it was not observed in the the mixture after ligation (lane 3 in Fig. 3). Futhermore it was clear that the sizes of DNA

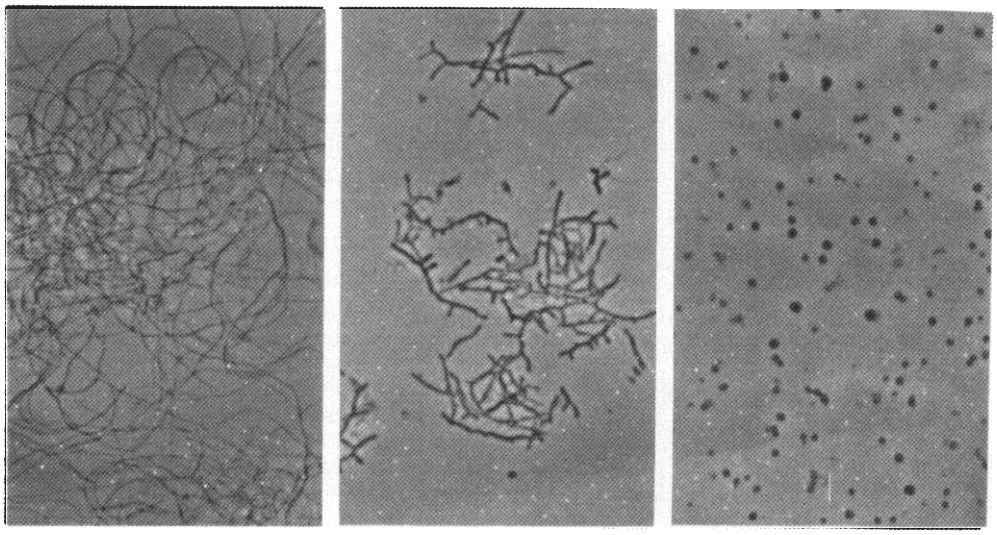

Fig. 2. *Protoplast formation of Streptomyces lividans 1326JI.*
(A) mycelia grown in a rich medium (YEME) without glycine. (B) mycelia grown in a rich medium (YEME) with glycine. (C) protoplasts obtained after lysozyme treatment

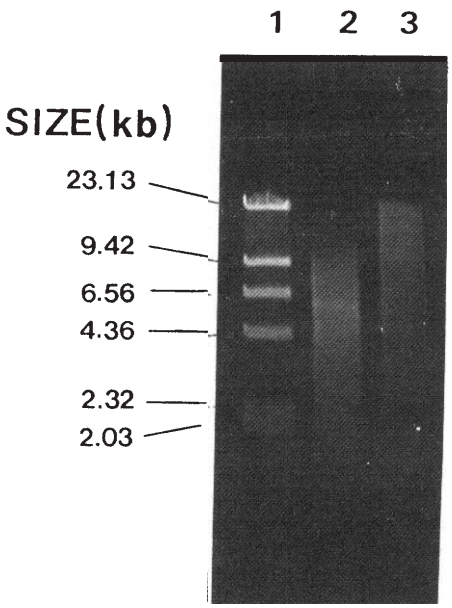

Fig. 3. *Ligation of Streptomyces sp. SMF-13 chromosomal DNA with plasmid pIJ702. The chromosomal DNA was digested with Sau3AI and the plasmid was digested with BgI II. The mass ratio of chromosomal DNA/vector DNA was 5/1. lane 1; φλ-HindIII, lane 2; ligation mixture before ligation, lane 3; ligation mixture after ligation.*

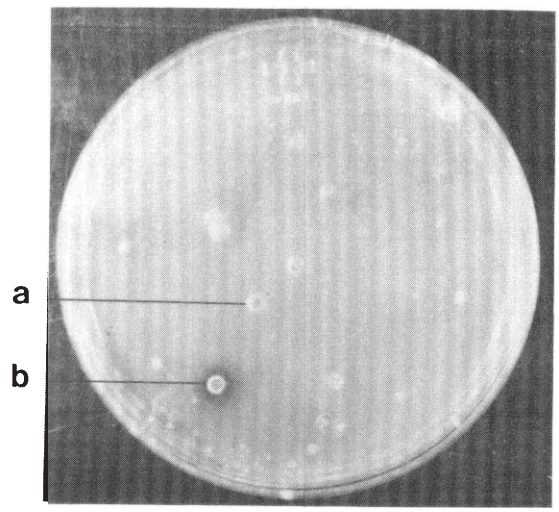

Fig. 4. *Regenerated cells of Streptomyces lividans 1326 JI on R2YE agar plate after transformation with ligation DNA mixtures.*
The black colony (ⓐ) indicates transformant harbouring self ligated plasmid pIJ702 and the white colony (ⓑ) indicates transformant harbouring recombinant plasmid DNA.

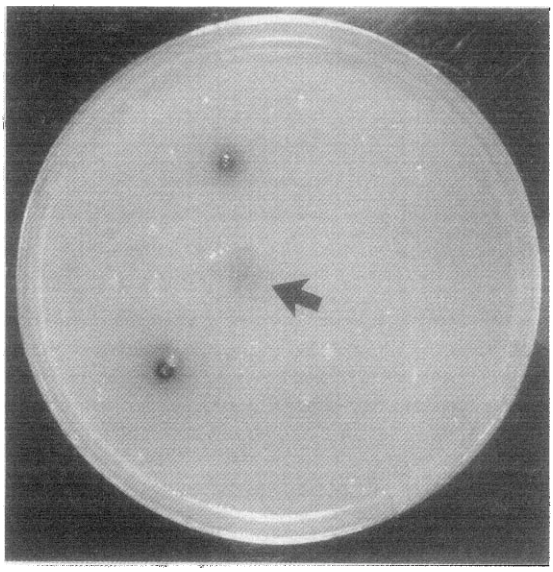

Fig. 5. *Screening of transformant producing extracellular β-lactamase.*
Transformants regenerated on R2YE agar plate were overlayed with nitrocefin solution (250 μg/ml) as described in context. The gray hollow zone formed around the colony (➘) indicates that nitrocefin was hydrolyzed by β-lactamase.

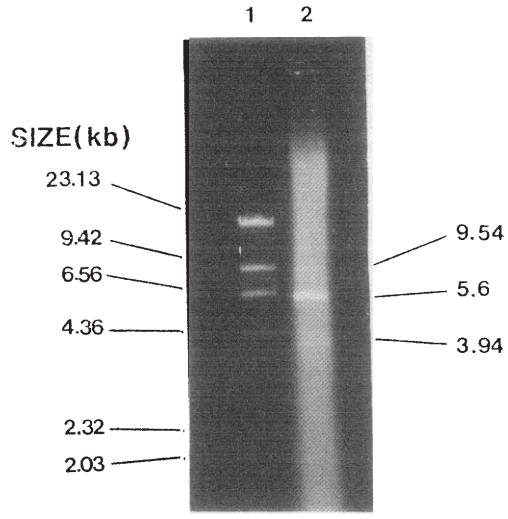

Fig. 6. *Mini-preperation of recombinant plasmid DNA isolated from the recombinant of Streptomyces lividans.*
Recombinant plasmid was partially digested with Bgl II. Plasmid pIJ702 (5.6 kb) and insert DNA (3.9 kb) were separated. lane 1; φλ-hindIII, lane 2; recombinant DNA partially digested with Bgl II.

fragments in the mixtures after ligation were greater than those before ligation. Those results indicated that DNA fragments were apparently ligated.

The ligation mixture was transformed to the protoplasts of *S. lividans* with the mediation of PEG 1000. And the cells were loaded on the R2 YE medium without thiostrepton and then incubated for 20 h at 30°C. The cells regenerated on the R2YE medium without thiostrepton were overlayed with hypertonic soft agar medium of the R2RE containing 250 μg/ml of thiostrepton. As results, colonies developed as white (ⓑ in Fig. 4) were selected as transformants having recombinant plasmid and the colonies developed as black were considered to be transformants having the vector DNA (ⓐ in Fig. 4). Total number of regenerated transformants per 1 μg of ligation DNA mixture was counted to be 2×10^3. However, that of regenerated transformants per 1 μg pIJ702 (CCC-form) was 1×10^6. The lower frequency of transformation at the ligated DNA mixture might be resulted from the changes in the DNA conformation. Among a thousand colonies regenerated on R2YE agar plate containing thiostrepton, one clony showed hydrolytic activity for nitrocefin and developed red hollow zone around it (Fig. 5). DNA fragment inserted on BglII site of plasmid pIJ702 in the recombinant was estimated to be 3.94 kb (Fig. 6). The β-lactamase activity in the culture broth of the recombinant strain obtained in this experiment was maximum to be 1.0 unit/ml after 3 days culture, which was very comparable to the donor strain (Table 1).

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초 록: *Streptomyces* sp. SMF13 균체외 β-Lactamase 유전자의 클로닝과 *Streptomyces lividans*에서의 발현

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균체외 β-lactamase의 donor strain으로 *Streptomyces* sp. SMF13을 선택하였다. 동 균주의 유전자를 Sau3A I으로 잘라 4 kb-10 kb 크기의 DNA 절편을 pIJ702의 Bgl II 위치에 cloning하여 *S. lividans*에 형질전환 하였다. 형질전환 시키기 위한 숙주균 *Streptomyces lividans*의 protoplast 형성과 regeneration의 조건을 확립하였다. 형질전환 효율은 $2\times10^3/\mu$g DNA였으며 삽입된 DNA 절편은 3.94 Kb임을 확인하였다. 형질전환 균주의 β-lactamase활성 은 3일간 배양후에 최대를 보였다(1.0 unit/ml)