EXTRACTION, CLONING AND PHYSICAL MAPS OF PLASMID DNAs FROM *STREPTOMYCES NOURSEI*

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Three plasmids, pSCY 2, pSCY 3 and pSCY 4, were detected in *Streptomyces noursei* B3, a producer of cycloheximide and nystatin. pSCY 3 and pSCY 4 had molecular sizes of 15.1 megadaltons (Md) and 3.2 Md, respectively. When covalently closed circular (ccc) DNAs were extracted from the mycelia during the course of cultivation, the yield of ccc DNA extracted per mycelium reached the highest value at the starting point of the exponential growth phase and decreased rapidly during exponential phase; the yield increased gradually in stationary phase. The smallest plasmid, pSCY 4, could not be detected after 42 hours of cultivation.

To purify and characterize DNA of the major plasmid pSCY 3, it was cloned into *Escherichia coli* using pACYC 184 as a vector, and the physical maps of the composite plasmids were constructed.

HAYAKAWA *et al.*[1] reported that cycloheximide-nystatin-producing *Streptomyces* sp. 2217-G_1 carried two plasmids, pSCY 1 and pSCY 2, with molecular sizes 3.2 megadaltons (Md) and 18 Md, respectively. This strain was later identified as *Streptomyces noursei* by SHIMAZU* (personal communication).

In the interest of developing a cloning system using *S. noursei* as host, we have examined the plasmid content of a variant strain (B3) and found two new plasmids, pSCY 3 and pSCY 4. We initially attempted to use the small plasmid, pSCY 4, as a vector, but its yield from the mycelia varied greatly with the age of the culture. This led us to examine the extraction yield of plasmids in the mycelia during the course of cultivation. Plasmid pSCY 3 was selected for development as a vector and was purified by cloning into *Escherichia coli*.

This paper describes the extraction and characterization of plasmids of *S. noursei* and physical maps of the composite plasmids of pSCY 3 and pACYC 184.

Materials and Methods

Strains, Plasmids, Culture Conditions and Plasmid Isolation

Streptomyces noursei strain 2217-G_1 was provided by Dr. K. SAKAGUCHI. Mycelia of strain 2217-G_1 forming gray aerial mycelium were treated with Polytron and then a strain forming white aerial mycelium, designated B3, was isolated. The strain B3 as well as original strain produced cycloheximide and nystatin, but plasmid yield in strain B3 was about 10 times more than that of the original strain. *Escherichia coli* WA 803 (hsd_K^-, met^-, gal^-, lac^-) obtained from Dr. ARBER, was used as the host for composite plasmid transformation. Plasmid pACYC 184[2] was supplied by S. N. COHEN.

For the isolation of *S. noursei* plasmids, strain B3 was inoculated into a 100-ml flask containing 25 ml of a seed medium: 0.2% glucose, 0.3% Polypepton, 0.4% yeast extract and 0.1% glycine (pH 7.2); this was incubated at 27°C for 2 days on a reciprocal shaker. The seed culture was inoculated at a 2% level to a 500-ml flask containing 100 ml of the same medium; incubation was again done at 27°C

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on a reciprocal shaker.

Isolation of the plasmids from *S. noursei* was carried out according to the procedure described earlier[8]. Composite plasmids from *E. coli* were prepared as described previously[4].

Construction of Composite Plasmids and Transformation

A mixture (0.9 μg DNA) of *S. noursei* plasmids consisting of pSCY 3 and a small amount of pSCY 2 was partially digested with *Bam* HI, and pACYC 184 (0.08 μg) was completely digested with the same enzyme. Each sample was heated at 70°C for 5 minutes, mixed and incubated with T_4 DNA ligase by the addition of 1/8 volume of ligation salts (300 mM Tris-HCl (pH 8.0), 40 mM $MgCl_2 \cdot 6H_2O$, 100 mM dithiothreitol (DTT), 3 mg/ml adenosine 5′-triphosphate (2Na)) at a total DNA concentration of 12.4 μg/ml. The ligated products were transformed into *E. coli* and transformants resistant to chloramphenicol (25 μg/ml) and sensitive to tetracycline (12.5 μg/ml) were selected. Construction of the composite plasmids was confirmed by agarose gel electrophoresis. The procedure for transformation of *E. coli* by $CaCl_2$ treatment has been described[4].

Digestion of DNA with Restriction Endonucleases

Plasmid DNAs (usually 0.5~2 μg/40μl) were digested with 3~5 units of enzyme at 37°C for 1 hour in the following buffers: A buffer (6 mM Tris-HCl (pH 7.6), 150 mM NaCl, 7.5 mM $MgCl_2 \cdot 6H_2O$, 6 mM mercaptoethanol (ME), 25 μg/ml bovine serum albumin (BSA)) for *Bam* HI; B buffer (6 mM Tris-HCl (pH 7.4), 60 mM NaCl, 11.5 mM $MgCl_2 \cdot 6H_2O$, 6 mM ME, 25 μg/ml BSA) for *Eco* RI, *Hin*d III, *Bcl* I and *Xba* I; C buffer (10 mM Tris-HCl (pH 7.4), 10 mM KCl, 11.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM DTT, 25 μg/ml BSA) for *Bgl* II and *Hpa* I, and D buffer (6 mM Tris-HCl (pH 7.4), 10 mM NaCl, 7.5 mM $MgCl_2 \cdot 6H_2O$, 6 mM ME, 25 μg/ml BSA) for *Kpn* I, *Sac* I and *Pvu* II. Digestion with two or more enzymes was done simultaneously or sequentially, depending on the salt concentration of the buffer required. The reactions were terminated by the addition of 10 μl of a solution containing 50% (v/v) sucrose, 100 mM ethylenediaminetetraacetate (EDTA (3Na)) and 0.025% bromophenol blue.

Agarose Gel Electrophoresis

Plasmid DNA and endonuclease fragments were analyzed by electrophoresis on a 0.7~1.0% agarose vertical slab gel (16×16×0.3 cm); this was run for 16~18 hours at 20 mA, using Tris-acetate buffer (50 mM Tris-HCl (pH 8.05), 20 mM sodium acetate, 2 mM EDTA (2Na) and 18 mM NaCl). After electrophoresis, the gel was stained with 0.1% ethidium bromide for 15 minutes and then photographed under 260 nm UV illumination.

Electron Microscopy

Plasmid DNA was prepared for electron microscopy as described previously[4]. The preparations were examined with a Philips EM 400 electron microscope, using DNA of pACYC 184 as internal standard.

Enzymes and Chemicals

Restriction enzymes and T_4 DNA ligase were obtained from Takara Shuzo Co. Ltd. (Japan), except for, *Hpa* I, *Bcl* I, *Xba* I and *Sac* I, which were purchased from New England Biolabs, Inc. Lysozyme was from Sigma. Pronase E was from Kaken Pharmaceutical Co. Ltd. (Japan). RNase was from BRL, and agarose was from Seakam Marine Colloids Co.

Results and Discussions

Extraction and Characterization of *S. noursei* Plasmids

Plasmids pSCY 2, 3 and 4 were detected in *S. noursei* B3. pSCY 3 and pSCY 4 have not been described previously. Plasmid yield over the course of cultivation of *S. noursei* was examined using a reproducible extraction procedure (Fig. 1). The total yield of ccc DNA increased steadily for the first 20 hours until the begining of the exponential growth phase, reaching 34 μg per 100 ml culture (20 hours). For the next two hours, which correspond to the exponential phase, the amount of ccc DNA remained almost constant (20~22 hours). When the growth of *S. noursei* B3 reached a maximum (24 hours),

Fig. 1. Quantitative changes in *S. noursei* plasmids during cultivation.

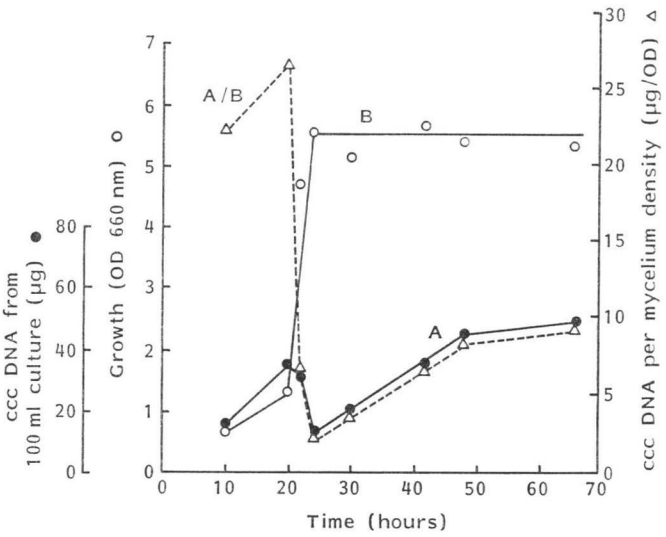
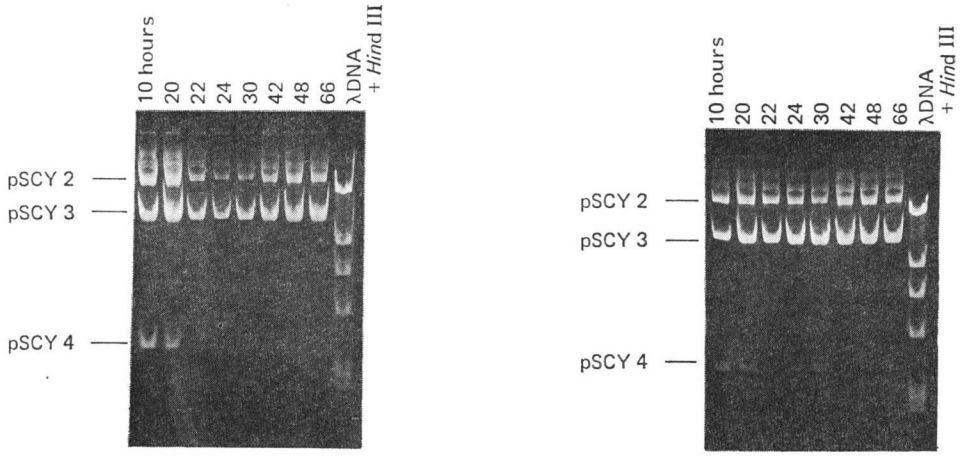


Fig. 2. Plasmid patterns of *S. noursei* at various stages of culture.



the yield of ccc DNA decreased, and then increased gradually again during the stationary phase of growth. The yield of ccc DNA was 58 μg after 66 hours. On the other hand, the yield of ccc DNA per mycelium density (OD 660 nm) reached its highest value at the starting point (20 hours) of the exponential growth phase. This value, however, decreased rapidly during exponential phase and then increased gradually again in stationary phase.

The change in the plasmid pattern of *S. noursei* B3 during cultivation was examined by agarose gel electrophoresis (Fig. 2). The patterns of fluctuation in the amounts of pSCY 2 and pSCY 3 were very similar, and their relative amounts were approximately the same at all sampling times. In all culture phases, pSCY 3 was present in the largest amount. By contrast, the smallest plasmid (in size and

amount), pSCY 4, was not detected after 42 hours of cultivation. The mechanisms of the variations in plasmid yield depending on the culture age may be considered as follows: extraction efficiency of plasmids, transition of plasmid forms among linear, open and closed circular DNAs, or changes in the integration and segregation to and from chromosomal sites.

The molecular size of pSCY 3 was determined to be 15.3±0.2 Md by agarose gel electrophoresis and 15.1±0.2 Md by electron microscopy; pSCY 4 was determined to be 3.2±0.2 Md by agarose gel electrophoresis. The molecular weight of pSCY 4 was similar to that reported for pSCY 1[1], but these two plasmids could be distinguished by restriction endonuclease analysis. pSCY 1 has a single site for *Bam* HI and no site for either *Hin*d III or *Eco* RI. In contrast, pSCY 4 had a single site for *Xba* I, 3 sites for both *Eco* RI and *Hpa* I, and 4 or more site for *Hin*d III. pSCY 3 had single sites for *Hpa* I and *Bcl* I, 2 sites for *Pvu* II, 3 sites for both *Sac* I and *Eco* RI, 4 sites for *Bgl* II and 5 sites for *Bam* HI. Plasmids pSCY 3 and pSCY 4 could not have been derivatives of the largest plasmid, pSCY 2, because pSCY 2 had no site for *Hin*d III and only a single site for *Bam* HI.

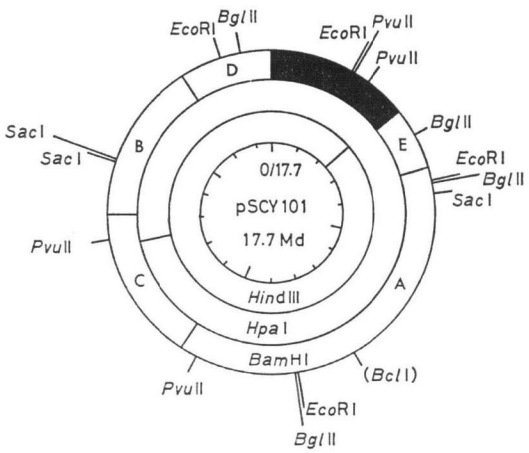
Cloning and Physical Maps of Composite Plasmids

pSCY 3 was selected for further investigation and to facilitate purification from the other plasmid(s), pSCY 3 was cloned into *E. coli*. A mixture of plasmids containing pSCY 3 was partially digested with *Bam* HI and ligated to *E. coli* plasmid pACYC 184 with T_4-ligase; this was transformed into *E. coli* WA 803. Colonies resistant to chloramphenicol and sensitive to tetracycline were selected and the plasmid DNAs were extracted and analyzed using restriction endonucleases.

Among 16 colonies tested were found to contain 9 types of composite plasmid. Among them, two types designated pSCY 101 and pSCY 102 contained the whole pSCY 3, whereas the other 7 types contained partial fragment(s) of pSCY 3.

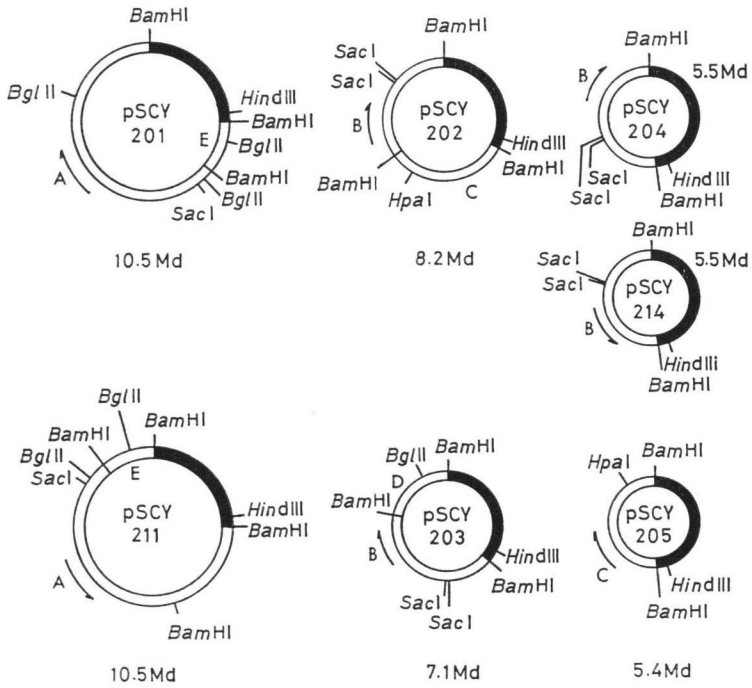
Fig. 3 shows the physical map of composite plasmid pSCY 101, in which 24 restriction endonuclease sites are mapped. pSCY 101 contained the *E. coli* vector between D and E of pSCY 3-*Bam* HI fragments. pSCY 101 carried single sites for the enzymes *Hin*d III and *Hpa* I and 6 sites for *Bam* HI. *Bcl* I cut the original plasmid, pSCY 3, at only one site in the *Bam* HI-A fragment, but this enzyme failed to cut the composite plasmid. Presumably the recognition site for *Bcl* I was modified in the *E. coli* strain used. The molecular size of pSCY 101 was estimated to be 17.7±0.3 Md by agarose gel electrophoresis and 17.8±0.3 Md by electron microscopy. These values were consistent with the value expected from the sum of the sizes of pSCY 3 and pACYC 184[2].

Fig. 3. Physical map of composite plasmid pSCY 101. Each division on the inner ring indicates 1.0 Md. The black area indicates pACYC 184.



Another composite plasmid, pSCY 102, differed from pSCY 101 only in the site joining the vector. pSCY 102 contained the pACYC 184 sequence between the B and D section of the *Bam* HI fragment. Seven types of composite plasmids, which contained a part of the *Bam* HI fragment(s) of pSCY 3, were also characterized. These results are summarized in Fig. 4.

Fig. 4. Composite plasmids between pACYC 184 and *Bam* HI fragment(s) of pSCY 3.
The black area indicates pACYC 184.



Preparation of a plasmid-free strain from strain B3, transformation system in that strain and pock-forming ability of pSCY 3 have already established in our laboratory (data not shown). By the use of these system, the above composite plasmids should prove useful for determining the replication region of *S. noursei* plasmid and in the construction of a shuttle vector for use with *S. noursei* and *E. coli*.

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