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Linear and Circular Conformation of Pock-Forming Plasmid pSA1 of Thiostrepton-Producing Streptomyces *azureus* ATCC 14921

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A wild-type strain (ATCC 14921) of thiostrepton-producing *Streptomyces azureus* is a pock-forming strain and harbors a plasmid pSA1. Plasmid pSA1 was found to be in a linear form in mycelia grown in abnormal plate culture with overlapping phage plaque-like pocks, but in a closed-circular form in liquid culture or in normal plate culture with no pock. The DNAs of both forms elicited conjugative pocks on sensitive strain PKC. The linear form of pSA1 was considered to *be* converted from the closed-circular form via an intermediate, an open-circular form. The copy size of closed-circular forms was one or less per host genome. The number of linear forms in one cell was estimated to be five or more. No plasmid was isolated from a mutant strain PKC, which lost the pock-forming activity.

INTRODUCTION

Many of the plasmids isolated from the *Streptomyces* species are phenotypically detectable according to their ability to elicit zones of growth inhibition, or "pocks", when the plasmid-carrying strains are grown in contact with plasmid-free strains on agar media. Plasmids SCP2 and SCP2* of *Streptomyces coelicolor* A3 (2) (Bibb *et al., 1977* ; Bibb and Hopwood, 1981) and several other *Streptomyces*' plasmids (Akagawa *et al.*, 1984; Kobayashi *et al.*, 1984; Manis and Highlander, 1982 ; Murakami *et al.*, 1983 ; Omer and Cohen, 1985) are such conjugative plasmids and elicit pocks.

Thiostrepton-producing *Streptomyces azureus* ATCC 14921 formed a unique lawn with phage plaque-like pocks on agar media (Ogata *et* al., 1981). These pocks appeared spontaneously during the growth of the organisms and increased in number in sub-culturing. This appearance brings about an abnormal growth of cultures, a diminished production of thiostrepton and difficulty for stock cultures (Ogate *et al.*, 1981). S. *azureus* also formed pocks (named conjugative pocks) similar to those of S. *coelicolor* and others on a sensitive strain PKC (Miyoshi *et* al., 1986). A low-copy number plasmid pSA1, which elicited pocks, was isolated from this wild-type strain. In our previous paper (Ogate *et al.*, 1983), pSA1 was found to be a linear plasmid in plate culture with overlapping pocks. This paper describes experiments concerning conversion from the circular to the linear form of pSA1.

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MATERIALS AND METHOD

Strains

Streptomyces azureus wild-type strain ATCC 14921 was used throughout this work. A pock-cured mutant PKC, previously called AF-6 (Ogate *et al.*, 1983), was obtained from wild-type strain by acriflavin treatment.

Medium

Rye flakes agar medium (Ogate *et al.*, 1981) was used for plate culture and pock formation. YEME medium supplemented with 34% sucrose and 5 mM $MgCl_2$ (YEMES) (Bibb *et al.*, 1977) was used as described below.

Plate culture with overlapping pocks

The plate culture of wild-type strain showed spontaneously developing plaque-like pocks. The pocks increased in number in serial subcultures. The incubation was for 3-5 days at 28°C.

DNA isolation

In the case of plate culture, spores of wild-type strain from abnormal slant or of strain PKC were inoculated and grown on cellophane-coated Rye flakes agar for 48 hr at 28°C. In the case of liquid culture, approximately 10^7 spores/ml of each strain were inoculated in YEMES medium and cultivated for 40 to 48 hr at 28°C on a rotary shaker (250 rpm).

Two g wet mycelia of each strain from the plate or liquid culture was adapted to DNA preparation. Total DNA and plasmid DNA from each strain were prepared as in Chater *et al.* (1982).

Estimation of copy numbers of plasmids

The relative copy numbers of plasmid in wild-type strain were estimated according to Kieser *et al.* (1982).

Digestion of DNA with restriction endonucleases and agarose gel electrophoresis

Digestion of DNA with restriction endonuclease *Eco*RI and agarose gel electrophoresis were carried out according to Maniatis *et al.* (1982). The molecular weights of DNA fragments were measured with reference to the *Hin*dIII digest of phage lambda DNA.

Hybridization

Hybridization was carried out by the Southern blotting method (Maniatis *et al,* 1982), as described previously (Ogata *et al.*, 1984).

Transformation

Protoplasting, regeneration and transformation were as described previously (Ogate *et al.*, 1983, 1985).

RESULTS AND DISCUSSION

Morphology of plate culture

Plate culture of wild-type strain shows spontaneously developing plaque-type pocks, as shown in Fig. l-b. Pocks in the plate increased in number during serial subcultures. After three or four transplantations, they became overlapping pocks or semiconfluent pocks, as shown in Fig. l-a.

We could temporarily cure this abnormality by the following treatment. Sores and mycelia of eroded plate were incubated in a liquid medium for more than 24 hr with shaking. After that, growing mycelia were reinoculated on Rye flakes agar. The lawn developed was characterized by a few pocks like that in the plate of Fig. l-b or occasionally no pocks. However, these cultures again became abnormal by serial subcultures.

Strain PKC, which was selected by acriflavin treatment, has no pocks, as shown in Fig. l-c.

Fig. **1.** Photographs of plate cultures of wild-type strain and strain PKC of *Streptomyces azureus*.
a, wild-type strain with overlapping pocks ; b, wild-type strain with few pocks ; c, strain PKC.

Isolation of plasmid pSA1 from mycelia grown in plate culture

The DNA preparation (cleared lysate) from the mycelia grown in plate culture was subjected to ethidium bromide-caesium chloride (EtBr-CsCl) density gradient centrifugation. The clear band of plasmid was hardly detected. Therefore, fractions obtained were loaded into the slots of an agarose gel, and Hind111 digest of phage lambda DNA was used as molecular size markers. As shown in Fig. 2, there is a distinct DNA band among bacterial DNA, of which the molecular length is approximately 9.0 kb. This band indicates a linear conformation.

The DNA preparation was next subjected to neutral sucrose density gradient centrifugation. Two DNA bands designated as A and B are observed, as shown in Fig. 3. The molecular lengths of bands A (major) and B (minor) are approximately 9.0 kb and 14.5 kb, respectively.

Separation and concentration were carried out on these two kinds of DNA. Figure 4 shows an agarose gel electrophoresis for each band DNA. It is confirmed that band A is about 9.0 kb and band B was about 14.5 kb. When each band DNA was applied

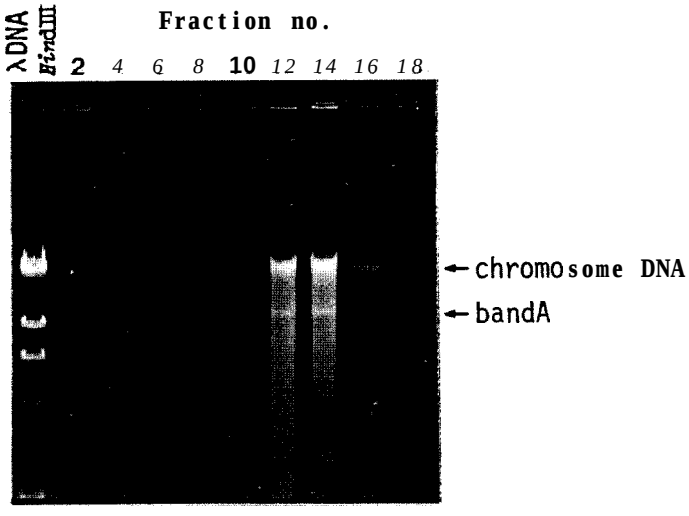

Fig. 2. Agarose gel electrophoresis of fractionated. DNAs from wild-type strain grown in plate culture of *Streptomyces azureus* after ethidium bromide-caesium chloride centrifugation.

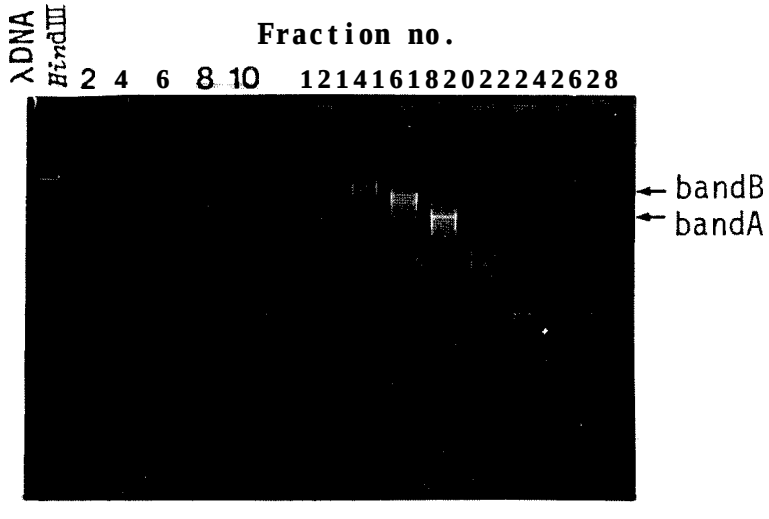

Fig. 3. Agarose gel electrophoresis of fractionated DNAs from wild-type strain grown in plate culture of *Streptomyces azureus* after sucrose density gradient centrifugation.

to EtBr-CsCl density gradient centrifugation, they were distributed at the position for linear conformation during centrifugation. On the other hand, these DNA bands were hardly detectable in the clear lysate from strain PKC.

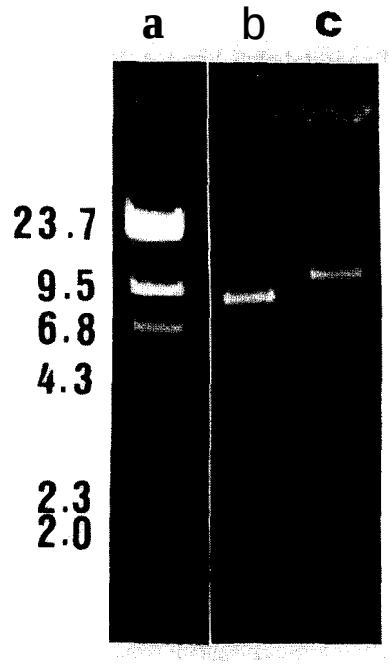

Fig. 4. Agarose gel electrophoresis of band A and band B DNAs from wild-type strain of *Streptomyces azureus*.
Lane a, phage lambda DNA digested with Hind111 ; lane b, band A ; lane c, band B.

During the purification of the DNAs of two bands with phenol-treatment, EtBr-CsCl density gradient centrifugation, agarose gel electrophoresis and so on, a part of band B DNA easily changed to band A DNA. This indicates that band B DNA has an open-circular (oc) conformation, which is nicked.

Isolation of plasmid pSA1 from mycelia grown in liquid culture

The same experiments as above were performed on the DNA preparation of the mycelia grown in liquid culture. A samll amount of closed-circular (cc) DNA band designated C was observed in EtBr-CsCl density gradient centrifugation. The molecular length was approximately 7 kb. During the preparation of band C DNA, it sometimes changed to two others having about 9.0 kb and 14.5 kb. From this result, it is supposed that band C DNA changes to band A DNA via an intermediate, band B DNA.

The relative copy number of plasmid pSA1 in the cells grown in liquid culture was estimated to be 0.5 to 1 per host genome throughout the whole growth phase. From this datum, the copy number of the linear forms in one cell grown in plate culture was calculated to be roughly 5.

The same circular plasmid was also isolated from the mycelia of normal plate culture with no pock or a few pocks like Fig. l-b. The copy number was less than 0.5.

Relationships among band A, band B and band C DNAs

To examine the similarity among bands A, B and C DNAs, Southern blot hybridization was carried out and radiolabeled band A DNA was used as a probe. A high

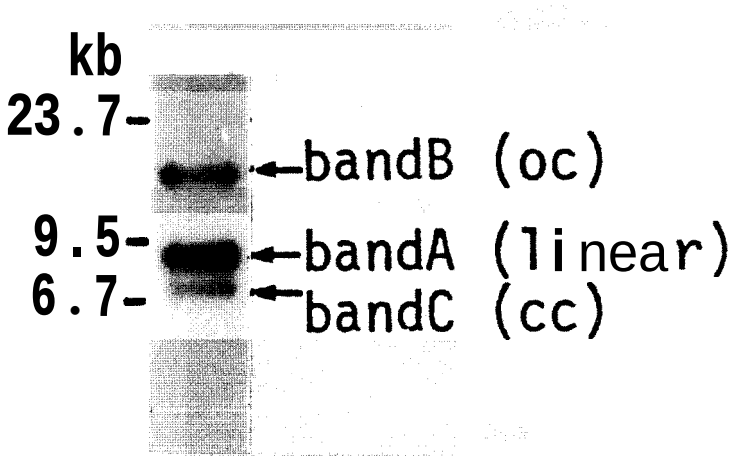

Fig. 5. Southern blot analysis of band A, band B and band C DNAs using band A DNA as a hybridization probe.
The lengths (in kb) and positions of *Hin*dIII-cleaved lambda DNA markers are indicated.

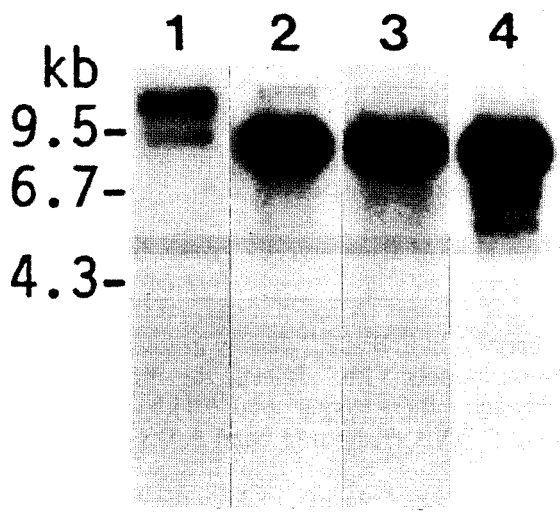

Fig. 6. Southern blot analysis of band A and band B DNAs using band A DNA as a hybridization probe.
Lane 1, band B DNA (with band A DNA) ; lane 2, *Eco*RI-cleaved band B DNA ; lane 3, band A DNA ; lane 4, *Eco*RI-cleaved band A DNA'.
The lengths (in kb) and position of *Hin*dIII-cleaved lambda DNA markers are indicated.

homology of these three DNAs was determined, as shown in Fig. 5.

To confirm the conversion of band B DNA to band A DNA, band B DNA was digested with restriction endonuclease EcoRI, and the digest was subjected to Southern blot analysis, as described above. Figure 6 shows that *Eco*RI-digest of band B DNA has the same molecular length (about 9.0 kb) as that of band A DNA. Untreated (control) sample of band B DNA was separated into two bands during its preparation or agar electrophoresis. The minor one corresponded to band A DNA. This result suggests that almost all band B DNA are converted to only band A (linear) DNAs by cleavage, and also band B DNAs has only one specific site for EcoRI.

EcoRI-digest of band A DNAs was separated into three or four fragments with a large quantity of undigested band A DNAs by agarose gel electrophoresis. This result suggests that the location of *Eco*RI-site on band A DNA may differ from molecule to molecule, and also the major part of band A DNA would not possess any EcoRI sites. From these results, it is considered that the conversion of band B DNA to band A DNA may be due to a specific or restricted cleavage and not by random cleavage. It is also supposed that the linear form of plasmid pSA1 in plate culture is produced from its original closed-circular form via an intermediate, open-circular form. The main conversion should occur *in vivo*.

Plasmid pSA1-free strain PKC

To examine the existence of pSA1 DNA in pock-cured strain PKC, DNA prepara-

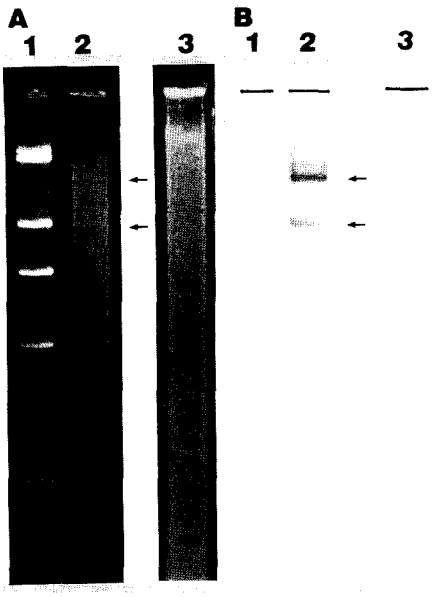

Fig. 7. Agarose gel electrophoresis and Southern blot analysis of DNAs from wild-type strain and strain PKC of *Streptomyces azureus*.
A, agarose gel electrophoresis; B, Southern blot analysis using band A DNA as a probe. Lane 1, *Hin*dIII-cleaved lambda DNA markers ; lane 2, wild-type strain DNA ; lane 3, strain PKC.

Table 1. Presence and conformation of plasmid pSA1 in pock-forming *Streptomyces azureus* ATCC 14921 and its pock-cured derivative.

Mycelia from plate culture of wild-type strain with overlapping pocks	Mycelia from liquid culture of wild-type strain	Mycelia from plate or liquid culture of pock-cured strain PKC
+ +		–
Linear (and OC)	CC	

+, Presence ; –, Absence ; OC, Open-circular ; CC, closed-circular

tion from strain PKC was applied to agarose gel electrophoresis together with DNA of wild-type strain. Then hybridization was carried out and radiolabeled band A DNA was used as a probe. As shown in Fig. 7, both bands (A and B) of wild-type strain hybridized very well with the probe. However, in the DNA preparation from strain PKC, there were no DNAs which hybridized with the probe band A DNA. This result indicates that pock-cured strain PKC has no plasmid pSAl and as such it is a plasmid pSAl-free strain. Table 1 summarized the presence and forms of plasmid pSAl in wild-type strain and strain PKC.

Pock-forming activity of linear and circular form DNAs

The pock-transforming activity of purified band A and band C DNAs was examined following the methods of protoplast transformation in the presence of PEG. About 1 x 10^7 protoplasts of strain PKC and 0.1 μg of both DNAs were used. Pock-forming transformants appeared at a low frequency with pSAl (approximately 1 to 5 × 10^3 pocks per μg of DNA), when they were plated together with strain PKC. Almost all colonies which were transformed continued to harbor the pock-forming activity even after several subculturing. This result indicates that linear and circular pSAl from a wild-type strain of S. *azureus* are associated with pock formation.

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