Characterization of a Plasmid from *Streptomyces coelicolor* A3(2)

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Covalently closed circular deoxyribonucleic acid (DNA) with a molecular weight of $20 \times 10^6$ was identified in strains of *Streptomyces coelicolor* A3(2) of various fertility types. Hybridization studies and digestion by various restriction endonucleases indicated that the circular DNAs (pSH1) were identical regardless of the fertility type (UF, IF, or NF) of the strain from which it was isolated. The pSH1 DNA was cleaved to many fragments by the endonucleases HincII, Smal, and SalI and to three or four fragments by BamHI and PstI. Plasmid pSH1 carries single sites for each of the two restriction enzymes, EcoRI and HindIII. These sites are $7.6 \times 10^6$ daltons apart. Attempts to isolate the fertility factor SCP1 as covalently closed circular DNA were unsuccessful. These data suggest that the biochemically isolated plasmid pSH1 is not identical to the genetically characterized fertility factor SCP1, which has been identified in an autonomous state in IF-type strains and in an integrated state in NF-type strains.

Previous genetic studies provided evidence that IF strains of *Streptomyces coelicolor* A3(2) contain a plasmid designated SCP1 (8). This plasmid acts as a fertility factor and carries genes for the antibiotic methylenomycin (18), which inhibits sporulation of UF strains of *S. coelicolor* lacking SCP1. The ability to synthesize methylenomycin can be efficiently transferred by conjugation to UF strains (16) or to strains of *S. lividans* (9). The SCP1 plasmid may be integrated into the chromosome of *S. coelicolor* strains of the NF fertility type (8). By mating IF strains with UF strains it has been also possible to construct *S. coelicolor* strains harboring derivatives of SCP1 in which various chromosomal segments of the donor strain have been inserted (9).

Our previous studies have shown that covalently closed circular (CCC) plasmid deoxyribonucleic acid (DNA) of about $20 \times 10^6$ daltons can be isolated from *S. coelicolor* A3(2) (12); in this report we describe the isolation of identical extrachromosomal DNAs from several strains of *S. coelicolor* with different fertility properties (IF, NF, or UF). No other CCC DNA that corresponded to the genetically described SCP1 plasmid could be detected by the biochemical procedures employed.

MATERIALS AND METHODS

Bacterial strains. *S. coelicolor* strains A3(2) (IF type), A332 pheAI (NF type), 1098 pheAI (UF type), 1190 hisA1, uraA1 strA1 (UF type), and 1984 hisA3 cysB+ SCP1 cysB+ and *S. lividans* strains 1326 (wild type), 1671 (SCP1), and 1923 cys-3 (SCP1 cysB+) were kindly provided by D. A. Hopwood.

Reagents. [methyl-3H]thymidine (specific activity, 24 Ci/mmol) was obtained from Radiochemical Centre, Amersham, England. Lysozyme, proteinase K and cesium chloride were purchased from Merck (Germany). Ethidium bromide was obtained from (Serva) Germany; agarose was from Seakem (USA).

Media. Minimal salt medium, complete medium (7), and sucrose-Casamino Acids-glycine medium (12) were used.

Growth of strains. Strains of *S. coelicolor* were grown on minimal-salt-agar slants until sporulation was completed. Spore suspensions in water were used to inoculate liquid medium.

Isolation of extrachromosomal DNA. Plasmid DNA was isolated from mycelial grown under vigorous shaking at 30°C for 40 h in 300 ml of sucrose-Casamino Acids-glycine medium that had been inoculated with about $10^6$ spores per ml.

Radioactively labeled extrachromosomal DNA was isolated after growth in sucrose-Casamino Acids-glycine medium containing 50 μCi of [methyl-3H]thymidine per ml (12). Mycelium was harvested by centrifugation and washed twice in TE buffer (0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.005 M ethylenediaminetetraacetic acid (EDTA), pH 7.5, containing 34% sucrose). A 2-g amount of mycelium was resuspended in 20 ml of the same buffer; 5 ml of 0.25 M EDTA (pH 8.0) and 60 mg lysozyme in 2 ml of TE buffer were added, and the mixture was incubated at 30°C. Formation of protoplasts, which was monitored by microscopy, occurred after 5 to 7 min. Sodium dodecyl sulfate, to a
concentration of 0.5%, and 200 μg of proteinase K per ml were added, and the mixture was kept for a further 30 min at 30°C. The concentration of sodium dodecyl sulfate was then raised to 1%, and the suspension was incubated for another 15 to 30 min at 30°C. Solid cesium chloride was then added to a final concentration of 1 M. The suspension was cleared by centrifugation as described previously (12).

**Dye-buoyant centrifugation.** A 14-ml amount of the cleared lysate, 0.5 ml of 0.25 M EDTA (pH 8.0), 0.5 ml of ethidium bromide (10 mg/ml), and 11.6 g of CsCl were mixed in a nitrocellulose tube of a Ti50-type rotor. Centrifugation was carried out at 20°C and 44,000 rpm for 40 h in a Beckman L5-65 centrifuge. Bands containing CCC DNA from three Ti60 gradients were pooled and rerun in a Ti50 gradient to equilibrium. Thus, the CCC DNA was concentrated to 50 to 100 μg/ml. From a lysate of 2 g of mycelium, 20 μg of CCC DNA could be obtained. After centrifugation in a cesium chloride gradient, the chromosomal DNA and CCC DNA appeared at the same density: 1.74 g/cm³.

Fractions containing CCC DNA were pooled, extracted with isopropanol to remove ethidium bromide, and dialyzed against cold 0.1 × SSC (0.015 M NaCl, 0.0015 M sodium citrate [pH 7.0]), and 0.0025 M EDTA). When required, CCC plasmid DNA was further purified by centrifugation on a linear 5 to 20% sucrose gradient in TES buffer (0.03 M Tris-hydrochloride, 0.005 M EDTA, and 0.05 M NaCl, pH 8.0). Centrifugation was performed at 20°C and 39,000 rpm for 80 min in an SW40 rotor.

**Electron microscopy.** Samples of 50 to 100 μl containing circular DNA were prepared for electron microscopy by the droplet method (1, 10).

**Hybridization studies.** DNA-DNA hybridization was performed by the membrane filter method (3) and as described earlier (4).

**Restriction endonucleases.** EcoRI and PstI were kindly provided by H. Mayer. HindIII was a gift from D. Blohm. HinCII was obtained from H. Lui brand. Smal was isolated from *Serratia marcescens* (15), and SalI was purified from *S. albus* as described by J. Groneberg. BamHI was isolated from *Bacillus amyloliquefaciens* H17.

**Assays for digestion of pSH1 DNA with restriction enzymes.** Cleavage of plasmid DNA with endonuclease EcoRI was performed in 25 mM Tris-hydrochloride (pH 7.5)-20 mM NaCl-10 mM MgCl₂ with HindIII, in 10 mM Tris-hydrochloride (pH 7.4)-10 mM MgCl₂-25 mM NaCl, with HindIII and PstI, in 10 mM Tris-hydrochloride (pH 7.5)-10 mM MgCl₂; with *Bam*HI, in 6 mM Tris-hydrochloride (pH 7.4)-100 mM NaCl-6 mM MgCl₂-6 mM 2-mercaptoethanol; with *Smal*, in 15 mM Tris-hydrochloride (pH 9.0)-8 mM MgCl₂-15 mM KCl; and with *SalI*, in 50 mM Tris-hydrochloride (pH 7.4)-40 mM NaCl-10 mM MgCl₂. Incubations were carried out in 50 to 100 μl at 37°C for 30 min to 4 h depending on the endonucleases. The reactions were terminated by the addition of sodium dodecyl sulfate and urea to final concentrations of 1% and 0.5 M, respectively.

**agarose gel electrophoresis.** Electrophoresis was performed in a slab gel apparatus constructed by D. Blohm. Gels contained 0.7 to 1% agarose in TAE buffer (0.04 M Tris, 0.02 M sodium acetate, 2 × 10⁻³ M disodium EDTA, and 0.018 M NaCl, adjusted with acetic acid to pH 8.0) (7). The samples were adjusted to 0.002% bromophenol blue and 12% sucrose and applied to the gel. Electrophoresis was performed at 2 to 6 V/cm at 4°C for 6 to 15 h. The gels were stained for 20 min in TAE buffer containing 4 μg of ethidium bromide per ml. The bands were visualized by fluorescence under ultraviolet light. Gels were photographed with an orange filter and HP, film.

**Molecular weight determination.** Molecular weights of the fragments were determined by coelectrophoresis of an EcoRI digest of λ DNA. The molecular weights of the EcoRI fragments of lambda were established previously (6).

**RESULTS**

Isolation of plasmid DNA from *S. coelicolor* strains having different fertility properties. CCC DNA could be isolated by cesium chloride-ethidium bromide centrifugation of cleared lysates prepared from UF, IF, and NF strains of *S. coelicolor* A3(2). The copy number of this extrachromosomal DNA is three to four per chromosome. The circular molecules represent a homogenous population of molecules with a contour length of 9.8 μm, independent of the strain from which the DNA was isolated (i.e., from a UF, IF, or NF strain). *S. coelicolor* strain 1984, which harbors a plasmid derived from SCP1 carrying the *cysB* region of the chromosome, was also analyzed for the presence of CCC DNA. Again, only a homogenous population of circular DNA of 20 × 10⁶ daltons or its multimers could be isolated (Table 1; Fig. 1). The *S. coelicolor* A3(2) strain 1984 produces the

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**Table 1. Physical properties of circular DNA molecules**

<table>
<thead>
<tr>
<th>Strain</th>
<th>s₂₀,w of supercoiled DNA</th>
<th>Copy no. per chromosome</th>
<th>Contour length (μm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1098 (UF type)</td>
<td>42-43</td>
<td>3-4</td>
<td>9.89 ± 0.2</td>
</tr>
<tr>
<td>A3(2) (IF type)</td>
<td>42-43</td>
<td>3-4</td>
<td>9.78 ± 0.2</td>
</tr>
<tr>
<td>1984 (SCP1 cysB+ type)</td>
<td>42-43</td>
<td>3-4</td>
<td>9.90 ± 0.2</td>
</tr>
<tr>
<td>A 332 (NF type)</td>
<td>42-43</td>
<td>3-4</td>
<td>NM a</td>
</tr>
</tbody>
</table>

*a* ColE1 DNA (2.04 μm) was used as an internal standard.

b NM, Not measured.
antibiotic methylenomycin. Individual colonies can be tested for this antibiotic production in a plate test involving inhibition of growth of the *S. coelicolor* UF strain 1190, which is initially present as a lawn of spores (9). Four isolates had spontaneously lost the ability to produce methylenomycin. These were also found to have lost the ability to grow without cystine. Both of these results indicated that the SCP1’ cysB+ plasmid had been lost. These segregants, obtained with a frequency of about 1%, showed no detectible loss of the 20 × 10^6-dalton plasmid (Table 2).

Strains of *S. coelicolor* A3(2) produce at least two bacteriocins that kill *S. griseus* 1157 and *Streptomyces* 1158 (D. A. Hopwood, personal communication). One UF strain, 2169, was isolated which had spontaneously lost the ability to produce the bacteriocin that kills *S. griseus* 1157. This strain had also retained the 20 × 10^6-dalton plasmid. In addition, crosses between the UF strain 2169 and the NF strain were performed. Analysis of the recombinants revealed that the ability to produce this bacteriocin is located on the chromosome next to the cysC,D locus (H. Schrempf, unpublished data).
Strains of *S. coelicolor* A3(2) normally contain a defective lysogenic prophage, φC31 (11). However, the $20 \times 10^6$-dalton plasmid is apparently not identical to the defective prophage since strains of *S. coelicolor* that had lost this prophage (UF 1889, φC31−; and IF 1890, φC31−) showed no detectable loss of CCC DNA. (Table 2)

Previous genetic studies have shown that SCP1 and SCP1' cysB+ plasmids could be transferred from *S. coelicolor* A3(2) to strains of *S. lividans* (9). The *S. lividans* strains thus obtained, 1671 and 1923, also synthesize the antibiotic methylenomycin as shown by their ability to inhibit the growth of the UF tester strain of *S. coelicolor* A3(2), 1190, or of the *S. lividans* wild type, 1326. In addition, a *S. lividans* cys-3 mutant regained the ability to grow in the absence of cystine when the SCP1' cysB+ plasmid of *S. coelicolor* 1098 was transferred into this mutant.

An attempt was made to isolate CCC DNA from these *S. lividans* strains by the same procedure as described before for *S. coelicolor*. However, no CCC DNA could be detected in the *S. lividans* wild type or in any of the transconjugants containing the SCP1' cysB+ plasmid.

In summary, these results indicate that with this technique it is not possible to isolate plasmid DNA that corresponds to the SCP1 plasmid from either *S. coelicolor* or *S. lividans* strains. However, plasmid DNA with a molecular weight of $20 \times 10^6$ can be isolated as covalently closed circles from all strains of *S. coelicolor*. The biochemical functions coded on this plasmid are still unknown. It does not seem to be involved in the determination of a bacteriocin active against *S. griseus* 1157, in methylenomycin production, or in the fertility exhibited by the *S. coelicolor* A3(2) wild-type strain.

Hybridization studies with the CCC DNA from various sources. Hybridization studies were performed to test whether plasmid DNA isolated from various strains of *S. coelicolor* A3(2) is indeed homologous. Various amounts of unlabeled circular plasmid DNA isolated from UF or IF strains were fixed on nitrocellulose filters as described previously (3, 4). The filters were incubated with 3H-labeled plasmid DNA of UF strain 1098. This DNA was degraded to fragments of about 8S by sonic oscillation and then denatured by heat. Figure 2 shows the saturation curves obtained. The amount of 3H-labeled DNA that bound to the corresponding unlabeled DNA of UF strain 1098 was taken as 100% homology. The hybridization of the labeled DNA with the DNA isolated from the other strains was related to this value.

The labeled DNA annealed, to the same extent, to filter-fixed plasmid DNA independent of whether it was isolated from UF strain 1098,
Previous genetic studies of Hopwood et al. (8) indicated that a transmissible plasmid, SCP1, distributed in these studies. If strains seem to contain SCP1 in an autonomous state. In UP strains, SCP1 is absent, and in NF strains, it is integrated in the chromosome. Furthermore, UP strains have been isolated in E. coli, as well as F factors, can be readily isolated from the plasmid. SCP1 can be genetically identified in UP and NF strains of S. coelicolor.

**Table 3. Molecular weights (×10⁶) of fragments after digestion of CCC DNA by various endonucleases**

<table>
<thead>
<tr>
<th>Endonuclease</th>
<th>EcoRI</th>
<th>HindIII</th>
<th>EcoRI/HindIII</th>
<th>PacI</th>
<th>PacI/EcoRI</th>
<th>PacI/HindIII</th>
<th>PacI/BamHI</th>
<th>BamHI</th>
<th>SalI</th>
<th>SalI/EcoRI</th>
<th>HindIII</th>
<th>HindII/EcoRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>~20.0</td>
<td>~20.0</td>
<td>~12.4</td>
<td>~10.2</td>
<td>~4.5</td>
<td>~11.5</td>
<td>~10.2</td>
<td>~6.0</td>
<td>~2.0</td>
<td>~3.0</td>
<td>~1.0</td>
<td>~2.0</td>
<td>~2.05</td>
</tr>
<tr>
<td>7.6</td>
<td>5.7</td>
<td>0.9</td>
<td>4.5</td>
<td>1.1</td>
<td>3.0</td>
<td>2.0</td>
<td>0.95</td>
<td>0.48</td>
<td>0.38</td>
<td>0.33</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>4.1</td>
<td>4.1</td>
<td>3.5</td>
<td>3.0</td>
<td>2.0</td>
<td>0.95</td>
<td>0.5</td>
<td>0.90</td>
<td>0.48</td>
<td>0.38</td>
<td>0.33</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>0.6</td>
<td>~1.3</td>
<td>(double band)</td>
<td>0.5</td>
<td>0.40</td>
<td>0.44</td>
<td>0.40</td>
<td>0.40</td>
<td>0.49</td>
<td>0.35</td>
<td>0.35 and several smaller fragments</td>
<td>~2.05</td>
<td>2.05</td>
</tr>
</tbody>
</table>

This enzyme is therefore useful for recognizing the possible molecular weight of the CCC DNA. Although a perfect comparison of the HindIII digests of CCC DNA is possible, it is not possible to cleave CCC DNA with a perfect enzyme. This enzyme was used to cleave CCC DNA from all sources to separate the fragments (Table 3). HindIII digestion products were separated by agarose gel electrophoresis (Table 3). HindIII also cleaved CCC DNA from all sources to separate the fragments. The HindIII enzyme from the probe strain was used to separate the fragments.
lost the SCP1' cysB+ plasmid still contained the 2 × 10^7-dalton plasmid. On the other hand, no CCC DNA could be isolated from S. lividans strains to which SCP1 or SCP1' cysB+ plasmids had been transferred. It can be concluded, therefore, that the plasmid isolated is not related to the fertility of S. coelicolor. To distinguish it from the genetically defined plasmid SCP1, the biochemically isolated plasmid was designated pSH1. Functions of pSH1 have not yet been identified. It does not represent the genome of prophage φC31 isolated from strain A3(2) and does not seem to determine the production of the antibiotic methylenomycin or the bacteriocin active against S. griseus 1157, since strains from which these properties have been

Fig. 3. Agarose gel electrophoresis of plasmid DNA cut by HincII (1, 3, and 4). Circular DNA was isolated from S. coelicolor strains of the UF type (1), IF type (3), or IF (SCP1' cysB+) type (4). EcoRI λ fragments (2). Electrophoresis was performed as described in the text.

lated as CCC DNA molecules by cesium chloride-ethidium bromide centrifugation of cleared lysates.

By the same technique, homogenous circular plasmid DNA of 2 × 10^7 daltons could be detected in several strains of S. coelicolor independent of their fertility type, i.e., IF, UF, or NF, which suggests that this plasmid is not related to SCP1. In addition, segregants of S. coelicolor SCP1' cysB+ that had spontaneously

Fig. 4. Sall digestion patterns of plasmid DNA isolated from S. coelicolor strains of the UF type (1), the IF type (2), and the IF (SCP1' cysB+) type (3).
employed, or may be a DNA molecule with a conformation other than the CCC type that is required for the isolation by the dye-buoyant density centrifugation technique.

DNA hybridization studies and digestion with restriction enzymes HindII, SmaI, and SalI suggest that plasmid pSH1 has the same nucleotide sequence regardless of its origin, which, furthermore, rules out the possibility that a plasmid of the same size but with different nucleotide sequences, and therefore different functions, may be present in *S. coelicolor* strains with different fertility properties.

Digestion of pSH1 with EcoRI and HindIII show that this plasmid has single sites for both enzymes, which are $7.6 \times 10^4$ daltons apart. It may be used, therefore, like RP4 in *Pseudomonas*, as a vehicle for studying transformation in *Streptomyces*. Although the location of the replication origin on the genome of pSH1 has not yet been determined, it appears quite possible, considering the BamHI or PstI physical map of pSH1, to construct from BamHI or PstI restriction fragments smaller plasmids that may be even more suitable as transformation vectors.

If properly handled, the described isolation procedure yields reasonable amounts of pSH1 DNA from *Streptomyces* strains for biochemical studies. However, we recently succeeded in joining pSH1 DNA to RSF2124(ColE1 Ap) and cloning this hybrid in *E. coli* (to be published). This interesting *E. coli*-*Streptomyces* hybrid plasmid can be amplified in *E. coli* by chloramphenicol to large amounts. The pSH1 part can then readily be cut out by EcoRI and separated from the ColE1 Ap part.

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**LITERATURE CITED**