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# Isolation of Covalently Closed Circular Deoxyribonucleic Acid from *Streptomyces coelicolor* A3(2)

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Covalently closed circular deoxyribonucleic acid (DNA) was isolated from two strains of *Streptomyces coelicolor* A3(2), representing two of the known fertility types. In each of the two strains circular DNA of about $20 \times 10^6$ daltons could be detected, amounting to about 1.5% of the total cellular DNA. The possible function of this DNA is discussed.

Extrachromosomal deoxyribonucleic acid (DNA) elements, which are either plasmids or defective phage genomes, have been isolated from a wide variety of bacterial species. However, as far as we are aware, no supercoiled DNA has been identified until now in streptomycetes. Genetic studies on *Streptomyces coelicolor* A3(2) have indicated that initial fertility (IF) strains harbor a plasmid designated as SCP1 (16, 17). This plasmid, as well as acting as a sex factor leading to mobilization of chromosomal markers (7), confers on strains that carry it the property of producing a diffusible substance that inhibits strains lacking the plasmid. The latter are designated ultrafertility (UF) strains. The SCP1 plasmid is transferred efficiently from IF to UF strains when they are grown together (16).

Although SCP1 of *S. coelicolor* A3(2) is genetically the best characterized plasmid among the streptomycetes, circumstantial evidence is accumulating for the control of several phenotypic properties by extrachromosomal elements in certain other streptomycetes; these include the production of melanin (5), aerial mycelium (12), and antibiotics (1, 12). Thus, plasmids may turn out to be important genetic determinants in this group of bacteria, as in the enterobacteria, pseudomonads, and staphylococci.

In this report the isolation of supercoiled DNA from both IF and UF strains of *S. coelicolor* A3(2) is described.

## MATERIALS AND METHODS

**Bacterial strains.** Strains 12 and 1098 of *S. coelicolor* A3(2), each carrying the chromosomal marker *pheA1*, were used for the isolation of DNA; the strains are of IF and UF fertility, respectively, 1098 having been derived from 12 after ultraviolet irradiation (17). Strain 1190, a UF strain bearing the chromosomal markers *hisA1 uraA1 strA1*, was used as indicator of the diffusible inhibitor produced by IF strains. Samples of mycelium from each culture used for DNA isolation were tested for inhibitor production, and so for presence of the SCP1 plasmid, by streaking on lawns of spores of strain 1190 on plates of "complete" agar medium (6). IF colonies gave rise to zones of nonsporulating and nonpigmented growth of 1190, whereas UF colonies did not (16). Spore suspensions for use as inocula for the production of liquid-grown mycelia were prepared from cultures grown on slants (6).

**Reagents.** [*Methyl*-$^3$H]thymidine (specific activity, 24 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. Lysozyme and proteinase K were purchased from Merck, Darmstadt, Germany. Ethidium bromide was obtained from Calbiochem, Los Angeles, Calif.

**Media for the growth of mycelium.** Minimal salts medium contained (per liter): glucose, 20 g; glycine, 2.6 g; monosodium glutamate, 2.2 g; $K_2HPO_4 \cdot 3H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.025 g; $CuSO_4 \cdot 5H_2O$, 0.012 g; $Mn\ SO_4 \cdot 7H_2O$, 0.016 g; $ZnSO_4 \cdot 7H_2O$, 0.030 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; and L-phenylalanine, 100 mg. The medium was adjusted with KOH to pH 7.2. Sucrose-Casamino Acids-glycine medium contained 34% sucrose, 2.4% Casamino Acids (Difco), 1% $MgCl_2$, 0.5% glucose, and 0.5% glycine.

**Lysis of the strains and isolation of supercoiled DNA.** Two methods of growth and lysis were used during this investigation.

(i) A 10-ml amount of minimal salts medium, supplemented with 10 μCi of [*Methyl*-$^3$H]thymidine per ml (24 Ci/mmol), was inoculated with spores and shaken at 30 C for 50 h. The mycelium was harvested by centrifugation at 15,000 rpm for 15 min at 4 C and washed twice with TES buffer (0.03 M tris(hydroxymethyl)aminomethane [Tris], 0.005 M ethylenediaminetetraacetic acid [EDTA], and 0.05 M NaCl, pH 8.0). The mycelium was then suspended in 1 ml of 0.01 M Tris-hydrochloride buffer (pH 7.3) containing 25% sucrose. To this suspension 0.3 ml of

0.25 M EDTA, pH 8.0, and 2 mg of lysozyme were added. $^{32}$P-labeled supercoiled ColE1 DNA was added as an internal control for nuclease activity during the lysis procedure. After incubation of the mixture for 30 min at 37 C, sodium dodecyl sulfate (SDS) to a concentration of 0.5% and 200 mg of proteinase K per ml were added, and the mixture was kept for a further 30 min at 37 C. Then the concentration of SDS was raised to 1% and the suspension was incubated for another 15 min at 37 C. Following the method of Vapnek and Rupp (15), sodium chloride was added to a final concentration of 1 M. The suspension was kept in ice for at least 2 h and then cleared by centrifugation at 16,000 rpm and 4 C for 15 min in a Beckman J21B centrifuge.

(ii) Alternatively, the mycelium was harvested from a 10-ml culture grown for 70 h in the sucrose-Casamino Acids-glycine medium in the presence of 10 $\mu$Ci of [$^3$H]thymidine per ml. The washed cells were suspended in 1 ml of 34% sucrose in TE buffer (0.01 M Tris-hydrochloride, 0.001 M EDTA, pH 8.0), and 0.2 ml of 0.2 M EDTA (pH 8.0) and 0.2 ml of a lysozyme solution (25 mg/ml in 0.01 M Tris-hydrochloride, pH 8.0) were added and incubated for 5 min at 30 C. The mixture was quickly cooled in ice, and 0.2 ml of EDTA (0.25 M) and SDS (final concentration, 1%) were added. Sodium chloride was then added as before, and the lysate was cleared in the same way.

**Dye-buoyant centrifugation.** A 2-ml amount of the cleared supernatant fluid, 0.5 ml of 0.25 M EDTA (pH 8.0), 1 ml of TES (pH 8.0), 0.5 ml of ethidium bromide (1 mg/ml), and 3.65 g of CsCl were mixed in a nitrocellulose tube for the Ti50 type rotor. Dye-buoyant centrifugation was carried out at 2 C and 42,000 rpm for 36 h using a Beckman L2-65B centrifuge. Fractions of 15 drops were collected from the bottom of the tube in small vials. An 0.02-ml amount of each fraction was spotted on filter-paper squares and assayed for radioactivity.

**Sucrose gradient centrifugation.** Fractions from cesium chloride gradients containing closed circular DNA were pooled and dialyzed against 0.1× SSC (0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0) and 0.0025 M EDTA for 50 min. Samples of 0.1 to 0.3 ml were layered on linear 5 to 20% sucrose gradients in TES buffer or on alkaline sucrose gradients which contained, in addition, 0.2 M NaOH and 0.7 M NaCl. After centrifugation at 20 C and 45,000 rpm in the SW50.1 rotor of the Beckman L2-65B centrifuge, 10-drop fractions were collected on filter-paper squares.

**Counting of radioisotopes.** The filter-paper squares were dried and washed with 10% trichloracetic acid, twice with ethanol, and finally with ether. The dried filters were put in vials containing scintillation fluid [5 g of 2.5-diphenyloxazole and 50 mg of 1,4-bis-[2]-(5-phenyloxazolyl)benzene in 1 liter of toluene] and counted in an Intertechnique liquid scintillation counter SL30.

**Electron microscopy.** Samples (100 $\mu$liters) containing circular DNA were dialyzed against 0.15 M ammonium acetate (pH 7.2) and prepared for electron microscopy by the droplet method of Lang and Mitani (10). DNA of bacteriophage PM2 was used as the internal length standard. Electron microscopy was performed with a Siemens I electron microscope, and photographs were analyzed as described earlier (2).

## RESULTS

The mycelium of *S. coelicolor* A3(2) can be effectively lysed by lysozyme and SDS. In the first experiments, mycelium, grown for 50 h in minimal salts medium, was treated with lysozyme for 30 min at 37 C and subsequently lysed in the presence of proteinase K first with 0.5% and then with 1% SDS. When supercoiled $^{32}$P-labeled ColE1 DNA (molecular weight, 4.2 $\times$ $10^6$) was present in the mixture during the lysis procedure, nearly all of the ColE1 DNA could be reisolated as covalently closed molecules.

In later experiments the increased sensitivity to lysozyme of *Streptomyces* mycelium grown in the presence of high concentrations of sucrose (7) and glycine (13) was exploited. The optimal concentrations of Casamino Acids, sucrose, and glycine were determined, the medium finally chosen being that described in Materials and Methods. Although cultures of *S. coelicolor* grow very slowly in this medium, mycelium, harvested after 70 to 80 h, could be lysed very quickly with SDS after treatment with lysozyme for only 5 min at 30 C.

Using a modification of the method of Vapnek and Rupp (15), the lysate produced by either of the two procedures was cleared by low-speed centrifugation. The supernatant, which contained 25 to 40% of the total cellular DNA, was centrifuged to equilibrium in a cesium chloride-ethidium bromide gradient. The IF strain and the corresponding UF strain of *S. coelicolor* A3(2) both contain a small amount of DNA banding at a position corresponding to a higher buoyant density than most of the extracted cellular DNA (Fig. 1A and 2A). The DNA of these denser bands was further analyzed by sucrose gradient centrifugation.

Both strains contain one species of DNA sedimenting at 42 to 43*S* under neutral conditions (Fig. 1B and 2B). Under alkaline conditions again only one DNA species sedimenting at 86 to 90*S* could be detected in each strain (Fig. 1C and 2C). Based on the data of Clayton and Vinograd (3), these sedimentation values indicate that this DNA represents supercoiled DNA with a molecular weight of about 20 $\times$ $10^6$. No larger covalently closed circular DNA could be detected when shorter centrifugation times were used or when the cleared lysates were assayed directly on neutral and alkaline sucrose gradients.

The slowly sedimenting peak in Fig. 1B and C

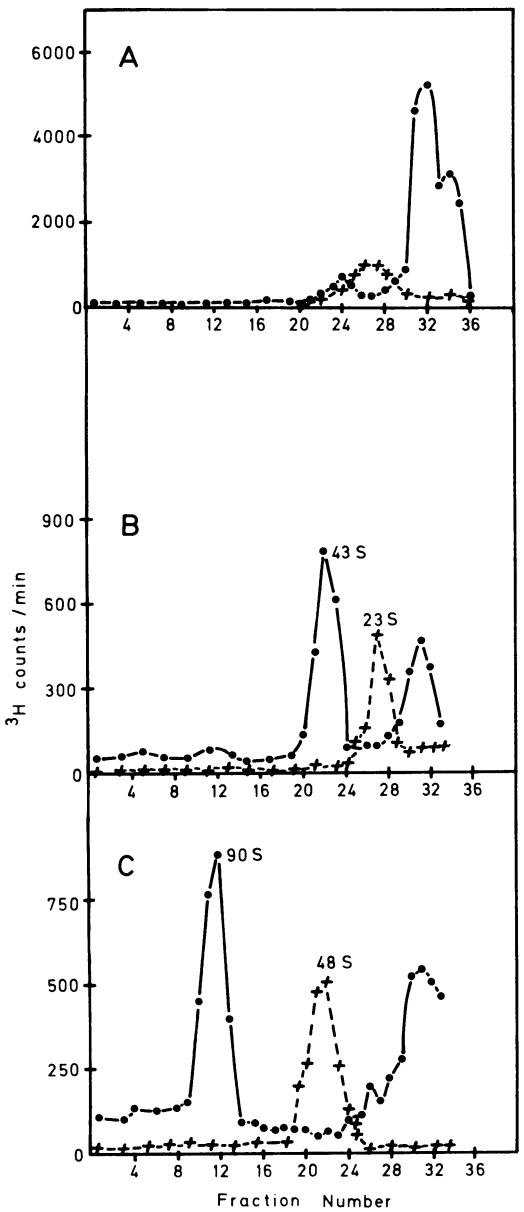


FIG. 1. *(A) Cesium chloride-ethidium bromide centrifugation of a cleared lysate of a 10-ml culture of S. coelicolor A3(2) strain 12 (IF type) grown in minimal salts medium in the presence of 10 μCi of [$^3$H]thymidine per ml. The lysate was prepared as described. After centrifugation in a cesium chloride gradient in the presence of ethidium bromide, fractions of 15 drops were collected from the bottom of the tube. Samples (0.02 ml) of each fraction were spotted on filter paper disks, which were assayed for radioactivity. (B) Neutral sucrose gradient analysis of the supercoiled DNA. The fractions of the denser DNA satellite band were pooled and dialyzed. An 0.2-ml amount was layered on a neutral 5 to 20% sucrose gradient and centrifuged at 45,000 rpm for 45 min at 20 C in an SW50.1 rotor using a Beckman L2-65 centrifuge. Fractions of 10 drops were collected from the bottom of the tubes on filter paper disks and assayed for radioactivity. (C) Alkaline sucrose gradient analysis of the supercoiled DNA. A dialyzed sample of the satellite DNA band was layered on an alkaline 5 to 20% gradient and centrifuged for 45 min under the same conditions as described in (B). S values given are related to supercoiled $^{32}$P-labeled ColE1 DNA, which was used as an internal marker and which sediments at 23S under neutral and 48S under alkaline conditions. Symbols: ●, $^3$H-labeled DNA of S. coelicolor; ×, $^{32}$P-labeled ColE1 DNA.*

does not represent supercoiled DNA. The quantity of these small fragments of DNA varies in different experiments and is sometimes present also in preparations from UF strains. This DNA probably represents small pieces of chromosonal DNA.

Electron microscopic analysis revealed that the isolated DNA molecules are indeed circular (Fig. 3). The molecules isolated from the IF strain have a contour length of 10.3 ± 0.3 μm and those of the UF strain 9.8 ± 0.2 μm, corresponding to molecular weights of 21.4 ± 0.6 × $10^6$ and 20.4 ± 0.4 × $10^6$, respectively (Table 1). Although these data indicate that the circular DNA from IF and UF strains differed slightly in size, further investigations would be necessary to confirm a difference.

The amount of supercoiled extrachromosomal DNA isolated was about the same whether the cells were lysed quickly after growth in the sucrose-Casamino Acids-glycine medium or whether the lysis procedure was performed over a longer period of time, as was necessary when the cultures were grown in the minimal salts medium.

The amount of extrachromosomal DNA represents about 1.0 to 1.5% of the total DNA in both strains. Taking the estimate of genome size for *S. coelicolor* of 5.2 × $10^9$ daltons (14), this amount would correspond to three to four copies of supercoiled DNA per chromosome and this would represent a minimal value as a loss of supercoiled DNA during the lysis procedure cannot be excluded. However, the estimate of genome size is so far based on a single report, the details of which have not yet been published.

## DISCUSSION

*S. coelicolor* A3(2) is the only representative of the streptomycetes whose genetics has been extensively studied (7). These genetic investigations have indicated that IF strains of *S. coelicolor* possess a transmissible plasmid (SCP1) which carries information for the production of

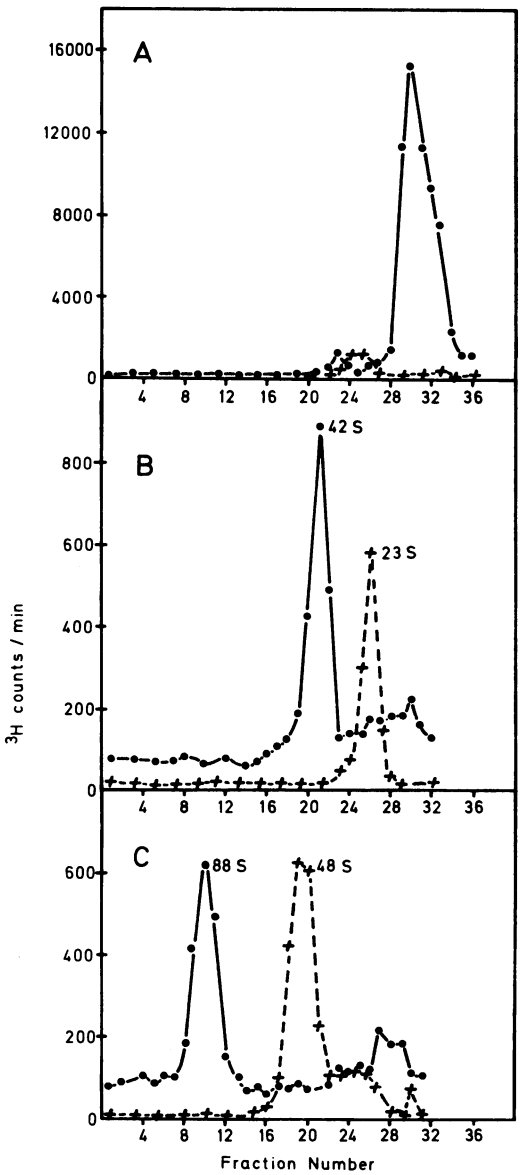


FIG. 2. *(A) Cesium chloride-ethidium bromide centrifugation of a cleared lysate of S. coelicolor A3(2) strain 1098 (UF type). (B) Neutral sucrose gradient analysis of the supercoiled DNA of strain 1098. (C) Alkaline sucrose gradient analysis of the supercoiled DNA of strain 1098. The centrifugation conditions and the symbols are the same as in Fig. 1.*

a diffusible substance that inhibits the growth and development of UF strains. These latter strains are interpreted as having lost the SCP1 plasmid. Plasmid loss, rather than mutation or deletion of plasmid genes without loss of the plasmid itself, is indicated by the rather frequent concomitant loss of a group of characters including inhibitor production, resistance to the inhibitor, and certain fertility properties. Moreover, the derived UF strains are readily reinfected with SCP1 by mixed growth with an IF strain, thereby regaining the full IF phenotype.

Covalently closed circular DNA molecules in the supercoiled configuration characteristic for all plasmids that have been isolated from *Enterobacteriaceae*, pseudomonads, streptococci, and staphylococci could also be isolated by a modified procedure from *S. coelicolor* A3(2). The circular DNA molecules isolated from an IF and UF strain have contour lengths of 10.3 ± 0.3 and 9.8 ± 0.2 μm, respectively, corresponding to molecular weights of 21.4 ± 0.6 × $10^6$ and 20.4 ± 0.4 × $10^6$. However, it was found that both the IF strain and the isogenic UF strain carry approximately the same amount of circular DNA of almost the same size. The amount is equivalent to about three to four copies per chromosome, assuming that the size of the chromosomal DNA of *S. coelicolor* is 5.2 × $10^9$ daltons (14). These findings make it unlikely that the circular DNA so far identified represents the SCP1 plasmid. No other plasmids were found in this study, although the method used should have allowed the isolation of plasmid molecules up to the size of 100 × $10^6$ daltons (unpublished data).

Several possibilities can be considered for the function of the circular DNA. (i) It may represent the genomes of spontaneously induced lysogenic or defective phages. A defective lysogenic phage, φC31, has in fact been demonstrated in A3(2) strains by Lomovskaya et al. (11), but its DNA has not been characterized. Although we failed to detect free phages in the supernatant of our cultures, the possibility cannot be excluded that the DNA circles represent genomes of phages that remain inside the cell. (ii) The DNA may represent plasmids determining functions common to both IF and UF strains and different from the functions coded for by the SCP1 plasmid. (iii) It could conceivably represent SCP1 plasmids with the same physical properties in both strains but with altered genetic determinants in the UF strain. Although, as mentioned above, the genetic data favor an interpretation of plasmid loss for the origin of the UF from the IF strain, a mutational origin cannot be completely excluded. In this context it will be interesting to determine if the extrachromosomal DNA isolated from UF strains is indeed about $10^6$ daltons smaller than that of IF strains. (iv) In spite of its apparent homogeneity and the similarity in the amount of supercoiled DNA in both strains, the extrachromosomal DNA of the

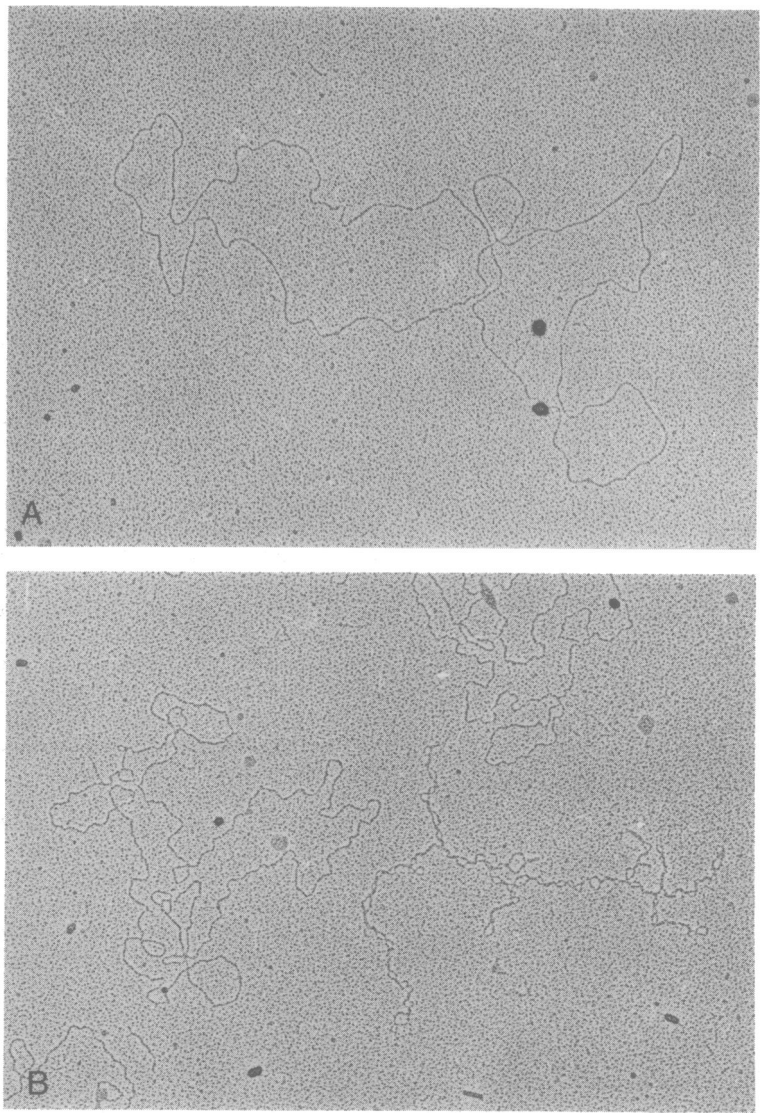


FIG. 3. *Electron micrographs of circular DNA. (A) Open circular molecule isolated from S. coelicolor A3(2) strain 12 (IF type). (B) Open circular and supercoiled molecules isolated from strain 1098 (UF type). Magnification, ×28,600.*

TABLE 1. *Contour lengths and molecular weights of circular DNA molecules isolated from strains of Streptomyces coelicolor A3(2)*

| DNA | Contour length (μm) | Mol wt (mega-daltons) | No. of molecules measured |
|---|---|---|---|
| Strain 12 (IF type) | 10.3 ± 0.3 | 21.4 ± 0.6 | 20 |
| Strain 1098 (UF type) | 9.8 ± 0.2 | 20.4 ± 0.4 | 38 |
| PM2 (*Pseudomonas* phage as internal length standard) | 3.22 ± 0.06 | 6.70 ± 0.13 | |

IF strain might possibly represent a mixture of SCP1 and a second plasmid, even a second sex factor, present in both IF and UF strains. This interpretation is consistent with the finding that chromosomal marker transfer occurs, at a low frequency, in UF × UF crosses (7). If the supercoiled DNA molecules represent or include the SCP1 plasmid of *S. coelicolor* A3(2), its physical size and hence its genetic information, would be considerably smaller than that of the fertility factor F of *Escherichia coli* (4).

The availability of strains of *S. coelicolor* A3(2), bearing derivatives of SCP1 in which chromosomal segments have been inserted (9), and strains of a different species, *Streptomyces lividans*, into which the SCP1 plasmid has been transferred from *S. coelicolor* A3(2), (8) should help to distinguish between these possibilities.

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