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Isolation and Physical Characterization of Streptomycete Plasmids

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Summary. Covalently closed circular DNA was isolated from a strain of *Streptomyces coelicolor* ATCC 10147 and from a strain of *Streptomyces coelicolor* subspecies *flavus* ATCC 19894, using two different methods. The two plasmids were of uniform monomer size: 8.9 kb for pS 10147, the plasmid from S. *coelicolor* ATCC 10147, and around 125 kb for the plasmid from S. *coelicolor* ATCC 19894.

A restriction enzyme map was constructed for pS 10147, using seven enzymes. Four of the enzymes, (*Bam*HI, *Bgl*,II, *Pvu*II, and *Xho*I) cut pS 10147 once while *Pst*I made two cuts. The GC content of this plasmid was calculated to be 72%. The possible utilisation of pS 10147 as a cloning vector in *Streptomyces* is discussed.

Introduction

Since the first report of possible plasmid involvement in antibiotic production in *Streptomyces* (Okanishi et al. 1970) and the first isolation of covalently closed circular DNA from *Streptomyces* (Schrempf et al. 1975), many investigations concerning Streptomycete plasmids have been undergone. Evidence of the role played by plasmids in antibiotic synthesis are accumulating (Hopwood 1978; Okanishi 1978). This evidence comes from genetic analysis, observation of the loss of antibiotic production after treatment with curing agents and isolation of plasmids in antibiotic-producing strains.

Reports of plasmid isolation have been published for S. *coelicolor* A 3 (2) (Schrempf et al. 1975; Bibb et al. 1977) for S. *kasugaensis*, which produces kasugamycin, (Okanishi 1977) for S. *venezuelae* sp 3022 a, which produces chloramphenicol, (Malik 1977; Malik and Reusser 1979), for a strain of S. *fradiae* ATCC 10745, which produces neomycin, (Yagisawa et al. 1978) for S. *puniceus, Streptomyces* sp 2217 C, *Streptomyces* sp 7068 CG, S. *hygroscopicus* (Hayakawa et al. 1979), for S. *lividans* (Bibb et al. 1980), and for S. *ribosidificus* ATCC 21294 (Nojiri et al. 1980). A restriction map has been established for SCP2 from S. *coelicolor* A3 (2), for pUC3 from S. *venezuelae* sp 3022a, for the series of SLP 1 plasmids from S. *lividans*. Among these plasmids, those of S. *coelicolor* A3(2) and S. *lividans* were used as cloning vectors in *Streptomyces* (Bibb et al. 1980; Thompson et al. 1980).

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In this paper we describe the isolation of plasmids from *Streptomyces coelicolor* ATCC 10147 and from *Streptomyces coelicolor* subspecies *flavus* ATCC 19894. The plasmid extracted from S. *coelicolor* ATCC 10147 was physically characterized, a restriction enzyme map for seven enzymes was established and the GC content of this plasmid was determined. The relatively small size (8.9 kb) and the four unique sites for different restriction endonucleases render this plasmid a suitable vector for DNA cloning experiments. As there is a problem of maintenance in interspecies plasmid transfer, this new plasmid might provide a tool to extend cloning to new *Streptomyces* species.

Materials and Methods

Bacterial Strains

Two strains of S. *coelicolor* from the ATCC were used; S. *coelicolor* ATCC 10147 and S. *coelicolor* subspecies *flavus* ATCC 19894.

Culture Media and Conditions

For sporulation both strains of *Streptomyces* were grown at 30° C on plates of Yeast extract agar (Pridham et al. 1957) consisting of Yeast Extract (Difco) 4 g/l, Malt Extract (Difco) 10 g/l, dextrose (Difco) 4 g/l.

Spore suspensions were used to inoculate liquid medium. Before DNA extraction *Streptomyces* strains were grown under constant shaking at 30° C in S. medium (Okanishi et al. 1974), supplemented with 1.25% glycine for S. *coelicolor* ATCC 10147 and 1.5% glycine for S. *coelicolor* ATCC 19894.

Isolation of Plasmid DNA from Streptomyces

Two methods were used to isolate plasmid DNA from *Streptomyces*: the first one (Bibb et al. 1977) tests the strains for the presence of plasmids and is used for the preparation of small amounts of plasmid DNA. The second method used to prepare larger amounts of plasmid DNA is derived from the method of Rush and Warner (1970).

Analytical Method

The mycelium cultivated in S liquid medium with glycine was harvested by centrifugation at 12,000 *g* for 20 min at 5° C, then washed with TES buffer (Tris-HCl 30 mM pH 8.0: EDTA 5 mM; NaCl 50 mM). TE buffer pH 8.0 (Tris-HCl 10 mM pH 8.0: EDTA 1 mM) containing 34% sucrose, 4.5 ml, was added to 0.5 g washed mycelium (wet weight). After treatment in a Potter homogenizer, 1 ml lysozyme (25 mg/ml) and 1 ml EDTA (0.25 M pH 8.0) were added to 5 ml homogenate and the mixture was kept at 30° C for 15 min. Then the addition of 1 ml EDTA (0.25 M pH 8.0) and 1 ml sarkoysl (20% w/v) caused lysis of the protoplasts. For each ml of lysate 1 g CsCl and 0.1 ml ethidium bromide (10 mg/ml) were added. Dye buoyant density centrif-

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ugation was carried out at 35,000 rpm for 60 h at 15° C in a Beckman Ti50 rotor.

Two bands were detected under ultraviolet (UV) light after centrifugation. The heavier band, corresponding to covalently closed circular (ccc) DNA was removed from the gradient by puncturing the side of the tube with a syringe. After extraction of ethidium bromide with butanol equilibrated with a saturated solution of cesium chloride, the DNA solution was dialysed against 0.1 SSC (1 SSC:0.15 M NaCl; 0.015 M Sodium citrate) to remove CsCl and then the DNA was precipitated with ethanol, after addition of NaCl to a final concentration of 0.3 M, and resuspended in TE pH 7.4 (Tris-HCl 10 mM pH 7.4; EDTA 1 mM).

Preparative Method

As in the analytical method, mycelium grown in S medium containing glycine was harvested by centrifugation and washed with TES buffer. NaCl/EDTA buffer (NaCl 0.15 M; EDTA 0.1 M; pH 8.5) was added to 10 g washed mycelium to obtain a total volume of 50 ml. After treatment in a Potter homogenizer, 5 ml lysozyme (50 mg/ml) were added and the homogenate was kept at 30° C for 15 min. Concentrated sodium hydroxide was added to a final concentration of 0.14 M and SDS (20% w/v) was added to a final concentration of 1%.

In the presence of NaOH and SDS the protoplasts were lysed. The lysate was kept 15 min at room temperature. The pH was then adjusted to pH 7 with a solution of 10% acetic acid. Potassium acetate was added to a final concentration of 1 M and the mixture was kept on ice for at least 1 h to precipitate the protein-SDS complex. After centrifugation at 12,000 g for 20 min, the supernatant was decanted and gently stirred in the presence of 5 g prewashed nitrocellulose (Nitrocel S. Serva, Heidelberg F.R.G.) to adsorb single-stranded DNA and was left for 20 min at room temperature. The mixture was then put in a syringe and nitrocellulose was slowly pressed in the syringe to recover as much solution as possible. Two volumes of absolute ethanol were added and the solution was kept overnight at −20° C to precipitate DNA. The DNA precipitate, recovered by centrifugation, was resuspended in TE pH 7.4 containing 1% sarkosyl. A dye buoyant density gradient centrifugation was performed and the cccDNA was recovered from this gradient as described above.

Electron Microscopy

The plasmids were prepared for electron microscopy by the protein film technique as modified by Inman and Schnös (1970). DNA, 5 μg/ml in TEF buffer (Tris-HCl 0.1 M; EDTA 0.01 M; formamide 50% pH 8.5) containing 0.01% cytochrome c, was used for spreading. The preparation was rotary shadowed with platinum and examined with a Philips EM 301 electron microscope. The DNA molecules were photographed and their contour length was measured on photographs (final magnification factor 100,000) with a map measurer. DNA molecules of plasmid pBR 322 were used as an internal standard in some preparations.

Endonuclease Digest and Agarose Gel Electrophoresis

Restriction enzymes were obtained from BRL Rockville USA, except *XhoI* and *PvuII*, which were purchased from Biolabs, Beverly, USA. The enzymes were used under the conditions described by the manufacturers. Incubations were carried out in 20 μl and the reactions were terminated by the addition of 3 μl solution containing: Sucrose 34%; Sarkosyl 4%; Bromophenol blue 0.1%; EDTA 1.5 mM.

Electrophoresis was carried out according to Gerbaud et al. (1979). Molecular weights of DNA fragments were calculated from their relative mobility in a gel. DNA of bacteriophage λcI857 digested by *EcoRI* or *Hind*III (Philippsen et al. 1978) and bacteriophage ΦX174 RF DNA digested by *Hae*III (Sanger et al. 1978) were used as molecular weight references. The agarose concentration in gels varied from 0.6% to 2.5% according to the size of the fragments being determined.

Determination of the GC Content of Plasmid DNA

The GC content of DNA was calculated from its buoyant density and was also studied directly by high performance liquid chromatography (HPLC) of the bases present in a DNA hydrolysate.

a) DNA buoyant density determination.

The buoyant density of DNA was determined by the technique of Meselson et al. (1957). DNA of bacteriophage λ (buoyant density 1.710 g/cm^3) was used as an internal standard. The GC content of DNA was calculated according to Schildkraut et al. (1962).

b) HPLC analysis of DNA hydrolysate.

The DNA was hydrolyzed by formic acid as described by Bendich (1957). HPLC analysis was performed according to Wakizaka et al. (1979).

Results

Isolation of cccDNA from Streptomyces

Lysozyme efficiently converts to protoplasts cells of both strains grown in the presence of glycine. Different glycine concentrations ranging from 0.25% to 3% were tested and the concentrations chosen (1.25% for S. *coelicolor* ATCC 10147, 1.5% for S. *coelicolor* ATCC 19894) partially inhibited the growth but nevertheless allowed isolation of large quantities of lysozyme-sensitive mycelium after 72 h growth. With the first method of extraction, in which the lysate was used directly for density gradient centrifugation, a band of supercoiled DNA was observed for both strains.

It has been shown by Vinograd et al. (1968) that within a narrow range of pH, around 12.0–12.5, linear DNA is converted into single-stranded DNA molecules while cccDNA remains partially double-stranded. Preparative procedures for cccDNA isolation based on selective alkali denaturation, followed by adsorption of single-stranded DNA on nitrocellulose, have been used for different organisms.

The pH must be high enough to allow maximum denaturation of linear DNA, but not too high, to avoid formation of irreversibly denatured froms of cccDNA. By adding the same final concentration of NaOH to a cell suspension containing the same amount of mycelium per ml, a reproducible pH value was obtained. To determine the NaOH concentration that gives the right pH value, several concentrations ranging from 0.12 M to 0.18 M were tested. A concentration of 0.14 M was found to give a better yield of DNA. With both strains the alkaline extraction method gave good results and allowed the isolation of cccDNA. A yield of 250 μg plasmid DNA was obtained for 10 g mycelium (wet weight) of the strain ATCC 10147.

The plasmids of S. *coelicolor* ATCC 10147 and S. *coelicolor* subspecies *flavus* ATCC 19894 were named respectively pS 10147 and pS 19894.

Electron Microscopy

The cccDNA from both strains was examined by electron microscopy.

Examination of the preparation of plasmid DNA extracted from strain ATCC 19894 revealed the presence of large molecules of circular DNA. These molecules were of uniform size, their average contour length was about 42 μm. One of these molecules is shown on Fig. 1a. Since this plasmid is too large to be easily used as a cloning vector it has not been used for further study.

Examination of the plasmid pS 10147 showed only one class of circular molecules whose average contour length was about 3 μm (Fig. 1b). Some dimeric molecules were visible (Fig. 1b) but they represented only a very small fraction of all the molecules observed. For more accurate size determination, the *Escherichia coli* plasmid pBR322 with 4362 nucleotide pairs

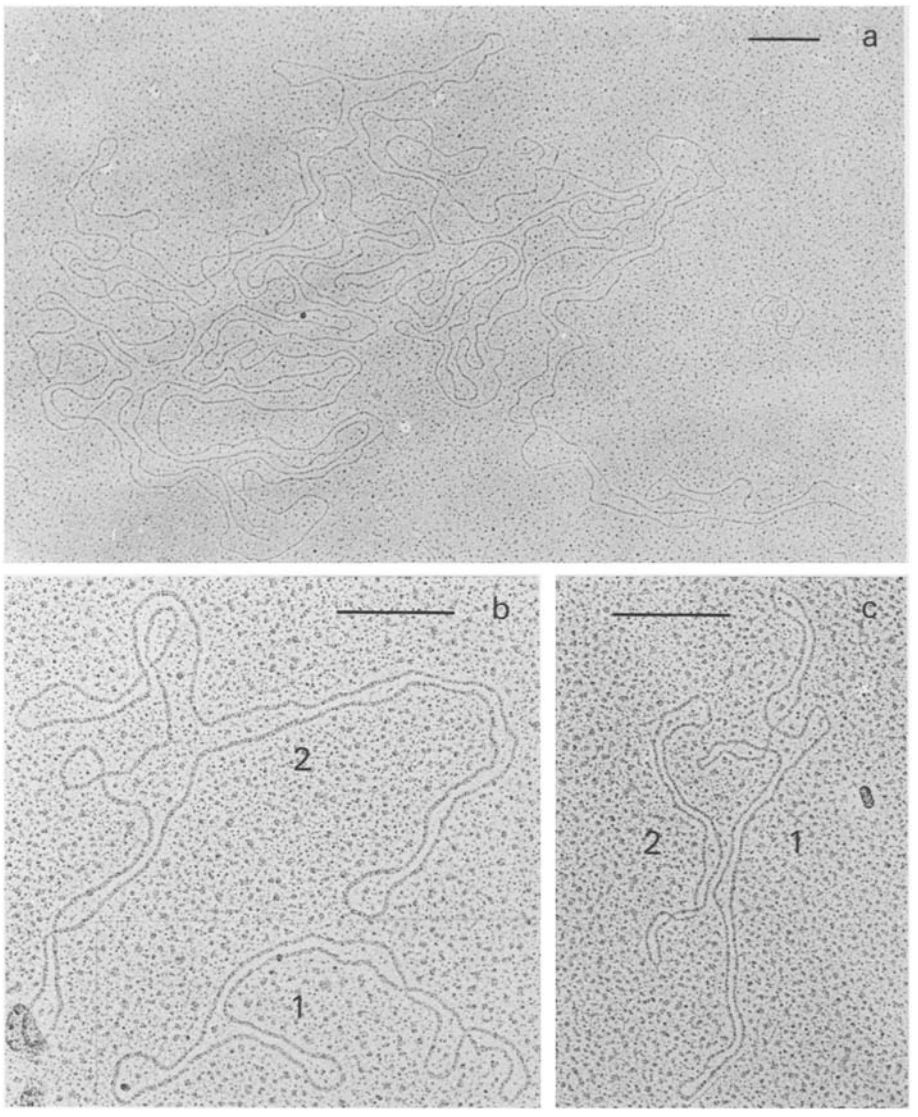

Fig. 1a–c. Electron micrographs of circular DNA molecules of **a** pS 19894, **b** pS 10147 monomer (1) and dimer (2), **c** a mixture of pS 10147 (1) and pBR322 (2) (Bar 0.25 μm)

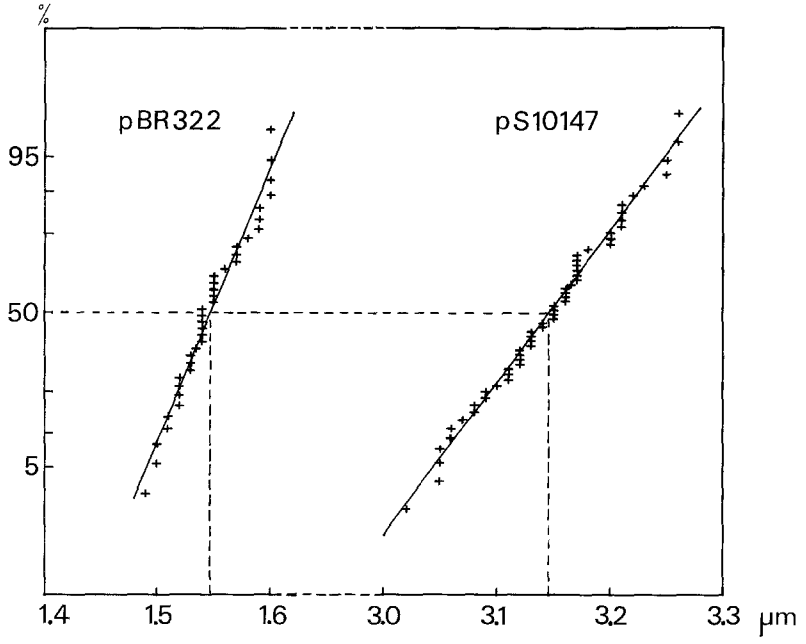

Fig. 2. Size distribution of pBR322 (left) and pS 10147 (right) Abscissa: contour length of open circular plasmid molecule (μm). Ordinate: cumulative Gaussian scale (%)

Table 1. Mapping of the *Bam*HI, *Bgl*II, *Pst*I, *Pvu*II, and *Xho*I sites

Enzymes	Size of the fragments (kb)	Total
*Bam*HI + *Bgl*II	6.15 2.80	8.95
*Bam*HI + *Pvu*II	5.00 4.00	9.00
*Bgl*II + *Pvu*II	6.80 2.20	9.00
*Bam*HI + *Bgl*II + *Pvu*II	4.00 2.80 2.20	9.00
*Bgl*II + *Xho*I	6.15 2.85	9.00
*Bam*HI + *Xho*I	8.85	8.90[a]
*Pst*I	5.45 3.45	8.90
*Pst*I + *Bam*HI	5.45 2.80 0.75	9.00
*Pst*I + *Bgl*II	3.45 3.40 2.05	8.90
*Pst*I + *Xho*I	5.45 2.75 0.80	9.00
*Bam*HI + *Bgl*II + *Pst*I + *Pvu*II	2.80 2.20 1.95 1.20 0.75	8.90

The sizes of large and small fragments were determined using different gels as described in Material and Methods

[a] The 0.05 kb fragment was not observed under the conditions used

(Sutcliffe 1978) was used as an internal standard. Open circular molecules of both plasmids were measured. One molecule of each plasmid is shown in Fig. 1c. The size distributions of these two plasmids were studied by tracing the probit curve (Fig. 2). A single homogenous population was observed for each plasmid. As the size distribution of the two plasmids is Gaussian, the average size corresponds to a cumulative frequency of 50%, giving 1.55 ± 0.01 μm for pBR322 and 3.15 ± 0.02 μm for pS 10147. The average contour lengths of pBR322 and pS 10147 are in a ratio that corresponds to a size of 8.86 ± 0.11 kb for pS 10147.

Endonuclease Digestion of pS 10147 DNA

In this first approach only endonucleases with recognition sequences of six base pairs were used. Others endonucleases with shorter recognition sequences might have yielded too many fragments for mapping at the projected scale.

No restriction site was found on pS 10147 for the endonucleases *Eco*RI, *Hind*III, *Hpa*I, *Sst*I, or *Xba*I. *Bam*HI, *Bgl*II, *Pvu*II and *Xho*I gave a single cut on pS 10147. Digestion of pS 10147 with *Pst*I generated two fragments. Four fragments were obtained with *Sst*II and seven with *Sal*I and *Sma*I. The total size of pS 10147 obtained by summation of restriction fragment sizes was consistent with the size obtained by electron microscopy.

Restriction Site Map

Table 1 contains the results of double or multiple digests, which allowed mapping of the restriction sites for *Bam*HI, *Bgl*II, *Pst*I, *Pvu*II, and *Xho*I. A typical gel is shown on Fig. 3.

A double digest with *Bam*HI and *Bgl*II generated two fragments of 6.15 and 2.8 kb. The *Bam*HI site was chosen as the origin and *Bgl*II site was arbitrarily assigned a position 2.8 kb from the *Bam*HI site in the clockwise direction. Double digests with *Bam*HI and *Pvu*II yielded two fragments of 5 and 4 kb, while double digest with *Bgl*II and *Pvu*II yielded two fragments of 6.8 and 2.2 kb. This allowed mapping of the *Pvu*II site 5 kb from the *Bam*HI site and 2.8 kb from the *Bgl*II site in the clockwise direction. This was confirmed by the triple digest with *Bam*HI, *Bgl*II, and *Pvu*II. A double digest with *Bgl*II and *Xho*I gave two fragments of nearly the same size as those generated by double digestion with *Bam*HI and *Bgl*II. Double digestion with *Bam*HI and *Xho*I generated one fragment slightly smaller than the linear form of pS 10147, the other fragment was not detected even with 2.5% agarose gel. These results show that the *Xho*I site is very close to the *Bam*HI site. The assignment of a precise position for the *Xho*I site will be possible later with the mapping of the *Pst*I sites.

*Pst*I treatment generated two fragments of 5.45 and 3.45 kb. The small fragment was cut by *Bam*HI into two fragments of 2.8 kb and a 0.75 kb. *Bgl*II cut the large *Pst*I fragment into two fragments of 3.4 and 2.05 kb. These results allowed assignment to a *Pst*I site a position 0.75 kb clockwise from the *Bam*HI

Table 2. Mapping of the *Sst*II sites

Enzymes	Size of the fragments (kb)			
*Sst*II	3.75	2.40	2.20	0.72
*Sst*II + *Bam*HI	3.75	(1.95 + 0.45)	2.20	0.72
*Sst*II + *Bgl*II	(2.05 + 1.70)	2.40	2.20	0.72
*Sst*II + *Pvu*II	3.75	2.40	(2.00 + 0.20)	0.72
*Sst*II + *Pst*I	3.75	2.40	(1.80 + 0.40)	(0.40 + 0.32)

The values in brackets are the sizes of the fragments generated by a cleavage in a *Sst*II fragment. The sizes of large and small fragments were determined using different gels as described in Material and Methods

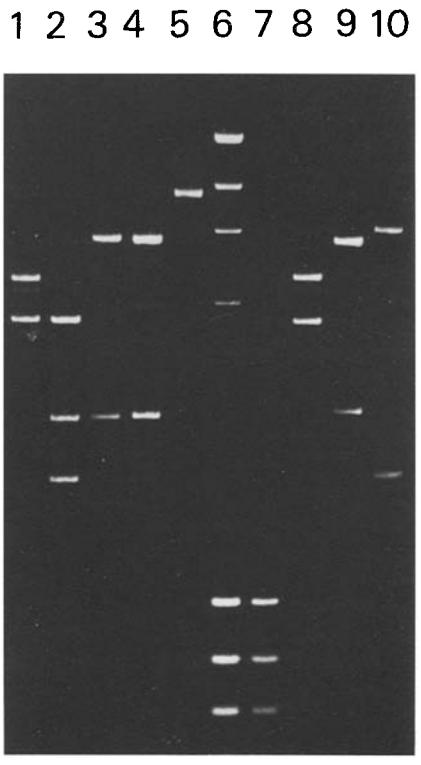

Fig. 3. Electrophoresis on 1% agarose gel: (1) pS 10147 digested with *Bam*HI and *Pvu*II; (2) pS 10147 digested with *Bam*HI, *Pvu*II, and *Bgl*II; (3) pS 10147 digested with *Bam*HI and *Bgl*II; (4) pS 10147 digested with *Bam*HI, *Bgl*II, and *Xho*I; (5) pS 10147 digested with *Bam*HI and *Xho*I; (6) Standard: λ DNA digested with *Hind*III and ΦX174 DNA, digested with *Hae*III (see Materials and Methods); (7) Standard: ΦX174 DNA digested with *Hae*III; (8) pS 10147 digested with *Pvu*II and *Xho*I; (9) pS 10147 digested with *Bgl*II and *Xho*I; (10) pS 10147 digested with *Bgl*II and *Pvu*II. Electrophoresis conditions were as described in Materials and Methods. Electrophoresis was from top to bottom

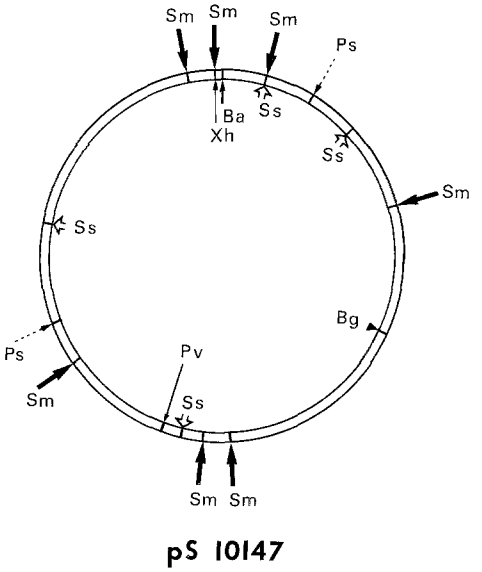

Fig. 4. Restriction endonuclease map of pS 10147. The abbreviations used code for the enzymes as follows; Ba: *Bam*HI, Bg: *Bgl*II, Ps: *Pst*I, Pv: *Pvu*II, Sm: *Sma*I, Ss: *Sst*II, Xh: *Xho*I. A least-squares method was used to provide the best fit for the coordinates of restriction sites. The computer program for carrying out this method was similar to that used by Schroeder and Blattner (1978). The coordinates are given from the *Bam*HI site in a clockwise direction: *Bam*HI 0/8.93 kb; *Bgl*II 2.74 kb; *Pvu*II 4.94 kb; *Xho*I 8.88 kb; *Pst*I 0.78 and 6.17 kb; *Sst*II 0.36, 1.07, 4.74, and 6.97 kb; *Sma*I 0.36, 1.86, 4.34, 4.62, 5.84, 8.69, 8.88 kb

site, the other *Pst*I site is 2.8 kb counterclockwise from the *Bam*HI site.

The size of the smallest fragment generated by the double digest with *Pst*I and *Xho*I is 0.8 kb. Compared with the 0.75 kb length of the smallest fragment in the *Pst*I and *Bam*HI double digest, it showed that the *Xho*I site is located 50 base pairs counter clockwise from the *Bam*HI site.

Some other double digests and digestion with *Bam*HI, *Bgl*II, *Pst*I, and *Pvu*II confirm the positions assigned to the six restriction sites.

A circular restriction site map of pS 10147 was drawn (Fig. 4). Double digests with *Sst*II and one of the other enzymes *Bam*HI, *Bgl*II, *Pvu*I, *Pst*I, and *Xho*I, whose sites have already been mapped, allowed assignment of map positions on the plasmid for the *Sst*II sites, as the four *Sst*II fragments are cut at least once by each of these five enzymes (Table 2). The same kind of experiments allowed mapping of the *Sma*I sites (Table 3) in spite of the fact that two of the seven fragments are not cut in double digests.

These sites are shown on the restriction site map (Fig. 4).

This method does not allow differentiation of the *Xho*I site from one of the *Sma*I sites, or the *Sst*II site, near *Bam*HI, from another of the *Sma*I sites.

Only three of the seven *Sal*I fragments were cut in double digests with *Sal*I and *Bam*HI, *Bgl*II, *Pst*I, *Pvu*II or *Xho*I (data not shown), this was not sufficient for mapping the *Sal*I sites by the double digest technique used for *Sst*II and *Sma*I.

GC Content of pS 10147

After analytical ultracentrifugation in a cesium chloride gradient, the plasmid gave a unique band whose buoyant density was

Table 3. Mapping of the *Sma*I sites

Enzymes	Size of the fragments (kb)						
*Sma*I	2.90	2.45	1.50	1.20	0.40	0.28	0.20
*Sma*I + *Bam*HI	2.90	2.45	1.50	1.20	(0.36 + 0.04[a])	0.28	0.20
*Sma*I + *Bgl*II	2.90	(1.59 + 0.86)	1.50	1.20	0.40	0.28	0.20
*Sma*I + *Pvu*II	2.90	2.45	1.50	(0.87 + 0.33)	0.40	0.28	0.20
*Sma*I + *Pst*I	(2.57 + 0.33)	2.45	(1.10 + 0.40)	1.20	0.40	0.28	0.20

The values in brackets are the sizes of the fragments generated by a cleavage in a *Sma*I fragment. The sizes of large and small fragments were determined using different gels as described in Material and Methods

[a] This fragment was not observed under conditions used

1.730 g/cm^3. This allowed calculation of a GC content of 72% according to Schildkraut et al. (1962).

As the GC content was very high, this result was checked by hydrolyzing the plasmid and determining directly the base composition using the HPLC technique. A value of 73% was obtained that was very close to the one estimated from isopycnic equilibrium centrifugation. So a GC content of 72% for pS 10147 can be assumed.

Discussion

The two strains harboring the plasmids described in this paper are not directly related to the strain of S. *coelicolor* A3 (2), which is to the geneticist the best known of the *Actinomycetes*. In fact S. *coelicolor* A3 (2) belongs to the species S. *violaceoruber* describted by Kutzner and Waksman (1959) as shown by Monson et al. (1969). But the strain ATCC 10147 is considered by Zähner and Ettlinger (1957) and by Kutzner and Wasksman (1959) to be a S. *violaceoruber*. So there may exist genetic homologies between the strain ATCC 10147 and S. *coelicolor* A3 (2).

Plasmid DNA was extracted from these two strains by two methods. These methods seem to be convenient: they allow isolation of plasmid DNA with quite a good yield; they appear not to break cccDNA because a very large plasmid was obtained. This large plasmid, pS 19894, was not studied because its size was an obstacle to its easy use as a cloning vector. To our knowledge, pS 19894 is the largest plasmid isolated from *Streptomyces*. Nothing is known about the genetic information carried by plasmids, pS 19894 and pS 10147.

The restriction enzyme pattern of pS 10147 shows that the enzymes whose recognition sequences comprise only G and C (*Sst*II and *Sma*I) frequently cut the plasmid. This is consistent with the very high GC content found for pS 10147 DNA. This result is in good agreement with the GC content found by Monson et al. (1969) for different strains of S. *coelicolor* and S. *violaceoruber* and with the 73% GC found by Bibb et al. (1977) for SCP2 DNA and S. *coelicolor* A3 (2) chromosomal DNA.

*Sal*I and *Sst*II are endonucleases extracted from *Streptomyces albus* Gl and *Streptomyces standford*; since pS 10147 is cut by these two enzymes it can be assumed that the restriction modification system of S. *coelicolor* ATCC 10147, if there is one, differs from the one in these two strains.

pS 10147 possesses characteristics that could be advantageous for cloning purpose. Its extraction is easy and large quantities of plasmid can be obtained. This may even suggest that pS 10147 is a multicopy plasmid. pS 10147 (8.9 kb) is smaller than SCP2, SLPI.2 or pUC 3. It has four unique restriction sites and two *Pst*I sites. Among the enzymes giving one cut, *Bam*HI and *Bgl*II yield fragments with the same cohesive ends. This could be used to delete a part of pS 10147. *Sau*3A, whose recognition sequence is only four base pairs, generates the same cohesive ends as *Bam*HI and *Bgl*II. So pseudo random fragments obtained by partial digestion with *Sau3*A could be cloned into pS 10147 using the *Bam*HI or *Bgl*II sites.

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