Determination of Methylenomycin A Synthesis by the pSV1 Plasmid from *Streptomyces violaceus-ruber* SANK 95570

By ALFREDO AGUILAR† AND DAVID A. HOPWOOD*

John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.

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A plasmid (pSV1) of 110×10^6 daltons from a methylenomycin A producing strain of *Streptomyces violaceus-ruber* was detected on and isolated from agarose gels. Elimination of this plasmid by protoplasting and regeneration resulted in the simultaneous loss of methylenomycin A production and resistance. pSV1 hybridized with pBR322 containing a cloned fragment of 1.7×10^6 daltons from *S. coelicolor* A3(2) which codes for methylenomycin A resistance. The pSV1 plasmid could be transferred to *S. lividans* by conjugation and by transformation and plasmid DNA identical in size to pSV1 could be isolated from the recipient strains. These experiments show that pSV1 codes for methylenomycin A production and resistance, in close analogy to the SCP1 plasmid from *S. coelicolor* A3(2).

INTRODUCTION

Methylenomycin A in *Streptomyces coelicolor* A3(2) is the only antibiotic whose specific biosynthetic enzymes are known to be coded by genes borne on a plasmid (Hopwood, 1979). This plasmid (SCP1) was first defined genetically by Vivian (1971), who characterized strains by their recombination properties as SCP1⁺ or SCP1⁻. SCP1⁺ strains were found to inhibit the growth of SCP1⁻ strains by producing a diffusible material; this was later shown (Wright & Hopwood, 1976) to be an antibiotic identical to methylenomycin A which had previously been detected in a strain of *Streptomyces violaceus-ruber* (Haneishi *et al.*, 1974*a, b*). The genetic evidence for the existence of SCP1 as a plasmid is substantial. SCP1 elicits lethal zygotis, a property associated with many *Streptomyces* plasmids (Bibb *et al.*, 1977, 1981). Fertility states can be recognized amongst derivatives of *S. coelicolor* A3(2) with the properties expected if a sex factor were absent (SCP1⁻), autonomous (SCP1⁺ or SCP1'), or chromosomally integrated (Hopwood *et al.*, 1973). The SCP1' strains (Hopwood & Wright, 1976*a*), analogous to F' strains of *Escherichia coli*, provide particularly strong evidence for SCP1 as an extrachromosomal element. SCP1' plasmids can be transferred by mating to *S. lividans* and *S. parvulus*; the latter, at least, has little chromosomal base sequence homology with *S. coelicolor* (Westpheling, 1980).

Evidence that structural, rather than regulatory, genes for the production of methylenomycin are carried on SCP1 is of two kinds. One arises from the ability to transfer SCP1 to other species. Thus *S. parvulus*, which normally produces the totally different antibiotic actinomycin D (Williams & Katz, 1977), makes methylenomycin A when SCP1 is mated into it (Kirby & Hopwood, 1977). More direct evidence is provided by the isolation of point mutations (*mmv*) eliminating methylenomycin A production in *S. coelicolor* A3(2) strains carrying SCP1 in the integrated (NF) or autonomous states (SCP1⁺ or SCP1'). All of the *mmv* mutations isolated are linked to SCP1 rather than to the chromosome (Kirby & Hopwood, 1977; Hornemann & Hopwood, 1981).

Physical data on the nature of SCP1 have been difficult to obtain, since the plasmid cannot be isolated by methods which readily recover most eubacterial and some other streptomycete plasmids (Westpheling, 1980). However, physical evidence for its existence came from differen-

† Present address: Departamento de Microbiología, Facultad de Biología, Universidad de León, Spain.

tial DNA hybridization experiments between SCP1⁺ and SCP1⁻ strains of *S. coelicolor* or between SCP1⁺ strains of *S. coelicolor* and *S. parvulus* (Hopwood *et al.*, 1979), together with some agarose gel electrophoresis studies (Westpheling, 1980). Okanishi *et al.* (1980) recently detected by electron microscopy a plasmid (pSV1) of 100 MDal in the original methylenomycin-producing *S. violaceus-ruber* strain. The present work describes the isolation of pSV1 and experiments which established that this plasmid, like SCP1, determines methylenomycin synthesis and resistance. This provides a step towards a detailed physical and genetic analysis of the methylenomycin system. A preliminary report of this work has been made (Aguilar & Hopwood, 1981).

METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. *Streptomyces violaceus-ruber* SANK 95570 was kindly provided by Dr Y. Kishida of the Sankyo Company, Tokyo, Japan. *Streptomyces lividans* strain M250, carrying plasmid pSLP111, was kindly provided by Dr M. J. Bibb.

Culture conditions and transformation procedures. Luria broth (Miller, 1972) was used for the cultivation of *E. coli* ED8767. For antibiotic selection of *E. coli* derivatives, tetracycline was added at 10 µg ml⁻¹ and ampicillin at 100 µg ml⁻¹. *E. coli* was transformed by CaCl₂ treatment (Cohen *et al.*, 1972). Genetic techniques for *Streptomyces* were those of Hopwood (1967) and Hopwood *et al.* (1973). For the isolation of plasmid DNA from *Streptomyces*, strains were grown in yeast extract/malt extract medium containing 1 M-sucrose and 5 mM-MgCl₂ (YEME) (Bibb *et al.*, 1977). For the preparation of protoplasts, mycelium was grown in YEME medium containing 10 mM-MgCl₂ (instead of 5 mM) and 66 mM-glycine. Transformation of *S. lividans* protoplasts, using polyethylene glycol, and protoplast regeneration, were as described previously (Bibb *et al.*, 1978) with slight modifications (Thompson *et al.*, 1982). The source of the pSV1 DNA used in transformation was a cleared lysate prepared as described below, before agarose gel electrophoresis. Selection for Mmy⁺ bacteria was done on complete medium (CM) agar (Hopwood, 1967) with plugs of agar containing a Mmy⁺ strain (Hopwood & Wright, 1976b).

DNA isolation, purification and analysis. Covalently closed circular (CCC) DNA was isolated from *E. coli* (Timmis *et al.*, 1978) and *S. lividans* (Bibb *et al.*, 1977) on ethidium bromide/caesium chloride density gradients. A mini-lysate procedure for analysis of plasmid DNA from *E. coli* and *Streptomyces*, used for screening purposes, was that of Birnboim & Doly (1979) with slight modifications (Chater *et al.*, 1982). pSV1 DNA was revealed in cleared lysates by the slightly modified version of Westpheling's (1980) procedure (Chater *et al.*, 1982). For the isolation of pSV1 from gels, only part of the gel was irradiated with short-wave ultraviolet (UV) light to locate the plasmid band. The plasmid DNA from the unirradiated part was electrophoresed into filter paper backed with dialysis membrane (Girvitz *et al.*, 1980), from which it was subsequently eluted.

Restriction endonucleases were obtained from Bethesda Research Laboratories and were used according to the manufacturer's instructions. Enzyme reactions were terminated by heating at 70 °C for 10 min and were monitored for completion by agarose gel electrophoresis. This was performed with horizontal 0.7% (w/v) agarose gels in either TBE (90 mM-Tris, 90 mM-boric acid and 1.3 mM-EDTA) or TAE (39 mM-Tris, 20 mM-sodium citrate and 1 mM-EDTA pH 8.3). After electrophoresis, the gels were stained in ethidium bromide (1 µg ml⁻¹) for 20 min and the DNA bands were visualized under UV light.

Transfer to nitrocellulose and hybridization. Plasmid and chromosomal DNA bands were transferred to nitrocellulose membranes essentially as described by Wahl *et al.* (1979) except that the gel was first irradiated with short-wave UV light (Mineralight lamp, model UVSL-58; Ultra-violet Products Inc., San Gabriel, California) from a height of 5 cm for 15 min (2.7 kJ m⁻²). Hybridization was performed in four times concentrated Denhardt's solution as described by Southern (1975) for 18 to 24 h at 65 °C. The filters were washed for 4 h in 2 × SSC (0.3 M-NaCl; 0.03 M-sodium citrate) at 65 °C. Autoradiography was done after preflashing the film (Fuji X-ray film). The film was exposed for 1 to 14 d at -70 ° using Ilford standard intensifying screens (CAWO, West Germany).

RESULTS

Antibiotic resistance and production by S. violaceus-ruber SANK 95570

Streptomyces violaceus-ruber SANK 95570 was resistant to the methylenomycin produced in solid medium by SCP1⁺ strains, as well as to pure methylenomycin A, to the same levels as *S. coelicolor* A3(2) SCP1⁺ strains. Thus, A3(2) and SANK 95570 were resistant to antibiotic discs containing 200 µg methylenomycin whereas 1190 showed inhibition zones at 20 µg. SANK 95570 inhibited the growth of SCP1⁻ strains of *S. coelicolor* and, to a lesser extent, the growth of SCP1⁺ strains. The inhibition produced on SCP1⁺ strains was evidently due to the production of another antibiotic (or bacteriocin) different from methylenomycin. Additional evidence for the existence of this second activity came from the fact that Mmy⁻ derivatives of SANK 95570, lacking pSV1, still produced another antibacterial activity (see below).

Table 1. Bacterial strains

Strain (JI stock no.)	Genetic markers	Plasmid status	Methylenomycin status	
			Mmy ⁺ /Mmy ⁻	Mmy ⁺ /Mmy ^s
<i>S. violaceus-ruber</i> SANK 95570				
3034	—	pSV1 ⁺	+	R
AA1	—	pSV1 ⁻	—	S
<i>S. coelicolor</i> A3(2)				
104	<i>hisA1 uraA1 strA1</i>	SCP1 ⁺ SCP2 ⁺	+	R
1190	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻ SCP2 ⁺	—	S
M124	<i>proA1 argA1 cysD18</i>	SCP1 ⁻ SCP2 ⁻	—	S
AA8	<i>proA1 argA1 cysD18</i>	SCP1 ⁻ SCP2 ⁻ pSV1 ⁺	+	R
<i>S. lividans</i> 66				
1326	—	—	—	S
1671	—	SCP1 ⁺	+	R
M250	—	pSLP111 ⁺	—	R
CT2	<i>str-1</i>	—	—	S
AA9	<i>str-1</i>	pSV1 ⁺	+	R
AA10	<i>str-1</i>	pSV1 ⁺ pIJ41 ⁺	+	R
<i>E. coli</i>				
ED87/67	<i>metB hsdS recA</i>	—

Fig. 1. Agarose gel electrophoresis of cleared lysates from *Streptomyces violaceus-ruber* SANK 95570 (track 5, with pSV1 arrowed) and from *Rhizobium leguminosarum* (tracks 1 and 2) and *R. phaseoli* (tracks 3 and 4). The strains (and molecular sizes of their plasmids in MDal) were as follows: Track 1, 897 (100, 165, 220); track 2, CO15 (100, 165, 195); track 3, 148b (130, 220); track 4, 4170 (130). The *Rhizobium* plasmid preparations were kindly provided by Dr G. Hombrecher and were obtained as described by Hombrecher *et al.* (1981). Plasmid sizes were estimated by N. J. Brewin, A. W. B. Johnston, G. Hombrecher, N. J. Dibb, J. W. Lamb and E. A. Wood (personal communication); plasmids larger than 220 MDal are ignored. The gel was 0.7% (w/v) agarose, with Tris/borate buffer and was run at 4 °C for 24 h at 3.3 V cm⁻¹.

Fig. 2. Plot of relative mobility vs molecular size of different plasmids used as standards. Plasmid standards of 100, 130, 165, 195 and 200 MDal come from Fig. 1. The estimated molecular size of pSV1 is about 110 MDal. The mobility of SCP2 (20 MDal) was related to that of pSV1 from other gels (not shown).

Detection and size of the pSV1 plasmid

Plasmid pSV1 could be detected in cleared lysates of SANK 95570 strains using the slightly modified version of Westpheling's (1980) procedure. In Fig. 1, pSV1 is seen together with several *Rhizobium* plasmids. From this photograph and others (not shown), using plasmids of known molecular weight, the size of pSV1 was estimated to be about 110 MDal (Fig. 2). The isolation method described for pSV1 was very reproducible, although the yield of plasmid DNA was low. All attempts to purify pSV1 from CsCl gradients failed. pSV1 eluted from gels had usually acquired nicks but could be cleaved with restriction endonucleases, including *Eco*RI and *Bam*HI.

Hybridization of pSV1 and S. coelicolor DNA to a probe containing the methylenomycin resistance gene of SCP1

To determine whether pSV1 has any homology with SCP1 we prepared a probe (pIJ601) consisting of pBR322 into which a 1.1 MDal *Pst*I-generated DNA fragment coding for methylenomycin A resistance (*Mmy*^r) had been inserted. The methylenomycin A fragment was originally cloned by Bibb *et al.* (1980) from an NF strain of *S. coelicolor* into SLP1.2 in *S. lividans*, replacing the region bordered by the *Pst*I sites and giving rise to plasmid pSLP111. pBR322 was chosen as a vector for the *Mmy*^r fragment of SCP1 in order to minimize the chance of any hybridization of SLP1.2 with *Streptomyces* chromosomal DNA [apart from the possibility of non-specific hybridization, homology with the integrated SLP1 sequences in the *S. coelicolor* chromosome would have been expected (Bibb *et al.*, 1981)].

Radioactively labelled probe DNA from pIJ601 had 10^6 to 10^8 c.p.m. ($\mu\text{g DNA}$)⁻¹. As shown in Fig. 3, pSV1 in lysates of SANK 95570 hybridized with the probe. Hybridization was also seen with the chromosomal DNA band of SCP1⁺ strain 104, suggesting that SCP1 had been linearized during (or before) the extraction procedure. Nevertheless, in *S. coelicolor* the hybridization was specific for SCP1⁺ strains (data not shown). These results strongly suggest that at least methylenomycin resistance is carried by the pSV1 plasmid. They also show that, after the isolation procedure which successfully reveals pSV1 as CCC DNA, SCP1 sequences are found in the linear chromosomal DNA fraction.

Fig. 3. Plasmid patterns (tracks 1 and 3) and corresponding autoradiograms, after hybridization with ^{32}P -labelled pIJ601 probe DNA (tracks 2 and 4), of *S. violaceus-ruber* SANK 95570 (tracks 1 and 2) and *S. coelicolor* 104 (tracks 3 and 4). The DNA patterns are displayed on 0.7% (w/v) Tris/borate agarose gels. Note hybridization of plasmid pSV1 (arrow) in track 2. In track 4, a fraction of the chromosomal DNA shows hybridization.

Stability of the Mmy⁺ phenotype of SANK 95570

About 1500 colonies of SANK 95570 on CM, at a density of about 100 colonies per plate, were replicated to *S. lividans* 1326 on CM as an indicator of Mmy production; no Mmy⁻ colonies were found. The same result was obtained from an approximately equal number of colonies arising from UV-irradiated spores (about 1% survival). However, when frozen protoplasts of SANK 95570 were regenerated on R2 medium and replicated to CM seeded with *S. lividans* 66, about 15% (11 out of 70) of the regenerated protoplasts were both Mmy⁻ and Mmy^s (Fig. 4). No plasmid DNA was detected in any of 10 of these isolates.

Fig. 4. Curing of methylenomycin A production (loss of pSV1) by protoplasting and regeneration of strain *S. violaceus-ruber* SANK 95570. These cultures were produced by replica-plating two master plates containing regenerated protoplasts of SANK 95570 on to lawns of *S. lividans* 1326, a Mmy⁺ strain. Several Mmy⁻ colonies can be seen (arrows).

Fig. 5. Transfer of pSV1 from *S. violaceus-ruber* SANK 95570 to *S. lividans* 66. Spores from a cross between SANK 95570 (Str⁺ pSV1⁺) and CT2 (*S. lividans* str-1) were replicated from R2 medium to CM medium containing 40 µg streptomycin ml⁻¹. Mmy⁺ transconjugants of *S. lividans* 66 were selected by their ability to grow in the zones of inhibition produced by plugs from a Mmy⁺ culture of SANK 95570. The Mmy⁺ transconjugants were found to be Mmy⁺ and to contain a plasmid identical to pSV1.

Transfer of pSV1

When spores from a cross of SANK 95570 and *S. lividans* CT2 (str-1) were plated on CM containing streptomycin (to counterscreen the donor), Mmy⁺ colonies of *S. lividans* were detected within the inhibition zone produced by plugs of an SCPI⁺ strain of *Streptomyces* (Fig. 5). Such Mmy⁺ strains (e.g. AA9) were found to produce methylenomycin and to contain a plasmid of the same size as pSV1 (Fig. 6, track 4). Similarly, pSV1-containing *S. coelicolor* strains (e.g. AA8) were isolated from a mating between *S. lividans* CT2/pSV1⁺ and *S. coelicolor* M124.

Plasmid pSV1 could also be transformed into protoplasts of *S. lividans* strain 1326. Using a cleared lysate from *S. lividans* strain AA10, carrying both pSV1 and pIJ41 (an SLP1.2 derivative carrying neomycin and thiostrepton resistances; C. J. Thompson, T. Kieser, J. M. Ward and

Fig. 6. Agarose gel electrophoresis of cleared lysates from different *Streptomyces* strains. Track 1, *S. lividans* 1671 (SCP1⁺); track 2, *S. coelicolor* 1190 (SCP2⁺); track 3, *S. lividans* AA10 (pSV1⁺, pIJ41⁺); track 4, *S. lividans* AA9 (pSV1⁺). The gel was run for 15 h at 3 V cm⁻¹.

D. A. Hopwood, unpublished results), pSV1 was transformed at a frequency 10^3 times lower than pIJ41, used as an internal control. pSV1 could be successfully isolated from Mmy⁺ Mmy^r transformants.

DISCUSSION

The involvement of plasmids in the production of antibiotics in *Streptomyces* has been suggested on many occasions, and recent reviews are available (Hopwood 1978, 1979; Okanishi, 1979). In many cases the evidence for plasmid involvement is indirect and often unsatisfactory. So far, the only example in which there is strong evidence that structural genes coding for antibiotic biosynthesis are plasmid borne is provided by methylenomycin A, coded by SCP1 in *S. coelicolor* A3(2) (Wright & Hopwood, 1976). Unfortunately there is little physical information on this plasmid.

The pSV1 plasmid, from a methylenomycin-producing strain of *S. violaceus-ruber*, was originally described, by electron microscopy, by Okanishi *et al.* (1980). We estimated its size as 110 MDal using several plasmids as standards, in reasonable agreement with the estimate of 100 MDal made by Okanishi *et al.* (1980). The fact that pSV1 hybridized with a probe containing the gene (or genes) coding for methylenomycin A resistance from *S. coelicolor* suggested that this plasmid might also be responsible for methylenomycin synthesis. This was indicated by the fact that about 15% of the regenerated protoplasts from *S. violaceus-ruber* were non-producers of methylenomycin and were Mmy^s. In all the cases examined, there was a concomitant loss of pSV1 DNA. Novick *et al.* (1980) and Hopwood (1981) reported that elimination of certain plasmids could be achieved during the formation and regeneration of protoplasts. The use of ethidium bromide, acriflavine and similar substances was avoided, because recent evidence (Schrempf & Goebel, 1979; Komatsu *et al.*, 1981) indicates that such so-called curing agents

often provoke DNA rearrangements, including deletions and insertions, instead of physical loss of plasmids. The involvement of pSV1 in methylenomycin production was confirmed by the finding that the plasmid could be transferred, by conjugation or transformation, to *S. lividans* 66 or *S. coelicolor* A3(2), whereupon the recipient strains became resistant to methylenomycin and also produced the antibiotic.

Plasmid pSV1 resembles the SCP1 plasmid in several respects, notably methylenomycin production and resistance, large size and transmissibility. However, they differ in some unknown way which makes SCP1 very difficult to isolate (Westpheling, 1980), in spite of its apparent similarity in molecular weight to pSV1.

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