Characterization of a Natural Cointegrate of the Pock-forming Plasmids pSK1* and pSK2* of *Streptomyces kasugaensis* MB273

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Strains carrying only one species of pock-forming plasmid, designated as pSK3*, were isolated from two different derivative strains of *Streptomyces kasugaensis* MB273 which contained three species of plasmids, pSK1, pSK2 and pSK3. Single and double digestion of pSK3* with seven restriction endonucleases yielded fragments identical with those of pSK3 and assignable to those obtained from pSK1* and pSK2*. In particular, digestion with *Bgl*II alone or in combination with other restriction endonucleases afforded the same size fragments as those of pSK1* and pSK2*. Strains containing pSK3* induced pocks on lawns of strains carrying pSK1* or pSK2* and resisted pock formation by the latter strains. Therefore, it was concluded that pSK3* was a pSK3 derivative with elevated pock-forming ability and represented a composite plasmid consisting of two elements, pSK1* and pSK2*, without any loss of their plasmid functions. Deletion derivative plasmids constructed from the *Bgl*II fragments of pSK3* provided evidence supporting the above conclusion. Pock formation by a pSK3*-containing strain against strains carrying pSK1*, or pSK2* or no plasmid accompanied the transfer of pSK3* from the former to the latter. Segregation of pSK1* and pSK2* from pSK3* was observed in mycelium from pocks caused by pSK3*-containing strains and on subculture of pSK3*-containing strains.

INTRODUCTION

Many conjugative plasmids have been isolated from *Streptomyces* species (Bibb *et al.*, 1977, 1981; Hopwood *et al.*, 1984; Akagawa *et al.*, 1984). This conjugative ability is often associated with the formation of clear zones or 'pocks' around colonies of the plasmid-carrying strain plated on the lawn of the plasmid-free strain ('lethal-zygosis' phenotype: Bibb *et al.*, 1977). Subsequently, pock formation was found to be useful in detection of transformants with these plasmid DNAs. Several useful vectors containing selective antibiotic resistance markers for cloning in *Streptomyces* have been constructed from pock-forming plasmids (Bibb *et al.*, 1980; Thompson *et al.*, 1980; Kieser *et al.*, 1982; Lydiate *et al.*, 1985).

Some of the conjugative plasmids, such as SCP2* plasmids, which arose as spontaneous segregants or mutants of the natural plasmid SCP2 (Bibb & Hopwood, 1981), and pIJ101 (Kieser *et al.*, 1982), have been reported to increase the frequency of genetic exchange between strains. For example, the SCP2* plasmid enhances chromosomal recombination between two strains of *Streptomyces coelicolor* by five orders of magnitude.

Streptomyces kasugaensis MB273 produces aureothricin and kasugamycin and harbours three species of plasmids, pSK1, pSK2 and pSK3. Recently, two spontaneous mutants of pSK1 and pSK2 with increased pock-forming ability (pSK1* and pSK2*) have been isolated from strains carrying pSK1 or pSK2 and characterized in terms of their unique endonuclease cleavage sites in DNA regions non-essential for pock formation (Akagawa *et al.*, 1984). In this report we describe the isolation of strains bearing only pSK3*, which also exhibit a high pock-forming ability, and appear to carry a composite plasmid consisting of pSK1* and pSK2*.

METHODS

Bacterial strains and media. *Streptomyces kasugaensis* strains used and generated in this study are listed in Table 1. GMY and MM agar media and GIPYG medium for submerged cultivation have been described previously (Akagawa *et al.*, 1984). MM was supplemented with histidine ($50 \mu\text{g ml}^{-1}$) or 1% (w/v) glucose instead of maltose, where appropriate. R3 and PWP media for protoplast preparation and regeneration were described by Shirahama *et al.* (1981).

DNA preparation, restriction endonuclease digestion, DNA ligation and agarose gel electrophoresis. These were performed as described previously (Akagawa *et al.*, 1984). Chromosomal DNA was prepared from a plasmid-free strain, A1R6. *Hind*III-digested phage λ DNA used as the size marker was purchased from Nippon Gene Co. Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Kaisha Co. or Boehringer-Mannheim Yamanouchi Co.

Detection of pock formation and plasmids. These were detected as described previously (Akagawa *et al.*, 1984).

Preparation, regeneration and transformation of protoplasts. *S. kasugaensis* protoplasts were prepared and regenerated as described previously (Akagawa *et al.*, 1984). Transformation was carried out as follows. To 0.1–2.0 μg DNA in 20 μl DSB (10 mM-Tris/HCl buffer, pH 7.6; 10 mM-NaCl; 1 mM-EDTA, disodium salt) in an autoclaved test tube (5 ml), 10 μl 50% (w/v) sucrose, 40 μl protoplasts (2.0×10^7) and 150 μl 30% (w/v) polyethylene glycol 2000 (Wako Pure Chemical Co.) in PWP were added successively and mixed. After 2 min on ice, the mixture was diluted with 1.7 ml PWP and then left for a further 5 min on ice. Samples (0.4 ml) of the diluted mixture were placed on R3 regeneration plates and mixed with 3.5 ml soft R3 medium ($40\text{--}42^\circ\text{C}$) containing 0.4% low-melting-point agarose (Bethesda Research Laboratories). Transformants were detected as described previously (Akagawa *et al.*, 1984).

RESULTS

Isolation of strains carrying pock-forming mutants of plasmid pSK3

As reported previously (Akagawa *et al.*, 1984), approximately 100% of viable hyphae of cultures of *S. kasugaensis* MB273 derivatives carrying pSK1* or pSK2* induced pocks (100% pocking efficiency) on a lawn of a plasmid-free strain, but the derivatives differed in their pock morphology. However, strain 18a, a spontaneous mutant of *S. kasugaensis* MB273 which contained mostly pSK3 with a reduced proportion of pSK1 and pSK2 DNA compared to MB273, showed only about 0.1% pocking efficiency on a lawn of the plasmid-free strain A1R6 (Akagawa *et al.*, 1984). More than 95% of these pocks exhibited a morphology similar to pSK1* pocks. Spores and mycelium from these pocks were picked to agar media and the resultant colonies were examined for plasmid content after growth in a liquid medium. All of 100 strains tested contained only one species of plasmid identical with pSK1. The other pocks were similar in morphology to pSK2* pocks. Nineteen out of 20 of these pocks provided colonies containing another species of plasmid identical with pSK2. However, one pock proved to contain a major species of plasmid equivalent to pSK3 together with two minor species of plasmids identical with pSK1 or pSK2. When colonies derived from spores and mycelium from 24 pocks induced by this strain were examined for plasmid content, a few of them were found to contain only one species of plasmid identical with pSK3. These strains exhibited 100% pocking efficiency and for this reason the plasmid was named pSK3*.

Two other strains carrying only pSK3 with high pock-forming ability similar to pSK3* were obtained from another strain, S1A1, carrying three species of plasmids, pSK1, pSK2 and pSK3. Among 200 colonies obtained after protoplasting and regenerating this strain, 20 were found to harbour pSK3 as the major species of plasmid and pSK1 and/or pSK2 as minor species. Two of these 20 colonies induced pocks on a lawn of the plasmid-free strain A1R6. Strains derived from 24 pocks from each of these two strains were examined for plasmids. One pock-derived strain from each of the two strains harboured only one species of plasmid, identical with pSK3 in restriction endonuclease cleavage patterns with seven different enzymes. Since these strains exhibited 100% pocking efficiency, their plasmids were regarded as pSK3* variants.

Table 1. *Strains of S. kasugaensis MB273 and their plasmids*

Strain	Remarks†	Plasmid(s)‡	Derivation§	Reference
MB273	Parent	pSK1/pSK2/pSK3 ^(m)	Spontaneous/MB273	Furumae <i>et al.</i> (1982)
18a	Amy ⁻ AT ⁻	pSK3/pSK1 ^(m) /pSK2 ^(m)	Regeneration/18a	
R5		pSK2/pSK3 ^(m)	Spontaneous/MB273	
S1	Amy ⁻	pSK1/pSK2/pSK3 ^(m)	AF mutagenesis/S1	
S1A1	Amy ⁻ Arg ⁻	pSK1/pSK2/pSK3 ^(m)	Regeneration/S1A1	Akagawa <i>et al.</i> (1984)
A1R6		Plasmid free	Pocks by MB273/A1R6 lawn	
A1R6(pSK1*)		pSK1*	Pocks by R5/A1R6 lawn	
A1R6(pSK2*)		pSK2*	NTG mutagenesis/A1R6	
R6N1	Mal ⁻	Plasmid free	NTG mutagenesis/A1R6	
R6N2	His ⁻	Plasmid free	Pocks by A1R6(pSK1*)/R6N1 lawn	
R6N1(pSK1*)	Mal ⁻	pSK1*	Pocks by A1R6(pSK2*)/R6N1 lawn	
R6N1(pSK2*)	Mal ⁻	pSK2*	Pocks by A1R6(pSK1*)/R6N2 lawn	
R6N2(pSK1*)	His ⁻	pSK1*	Pocks by A1R6(pSK2*)/R6N2 lawn	
R6N2(pSK2*)	His ⁻	pSK2*	Pocks by 18a/A1R6 lawn	
A1R6(pSK3*)	His ⁻	pSK3*	Transformation/R6N2/pSK3*DNA	This report
R6N2(pSK3*)	His ⁻	pSK3*	Transformation/R6N2/	
R6N2(pSK3*-D1)		pSK3*-D1	pSK3* DNA/BglIII/ligation	
R6N2(pSK3*-D2)	His ⁻	pSK3*-D2	As R6N2(pSK3*-D1)	
R6N2(pSK3*-D3)	His ⁻	pSK3*-D3	As R6N2(pSK3*-D1)	
R6N2(pSK3*-D4)	His ⁻	pSK3*-D4	As R6N2(pSK3*-D1)	
A1R6(pSK1*/pSK2*)		pSK1*/pSK2*	Pocks by A1R6(pSK1*)/A1R6(pSK2*) lawn	

† Amy⁻, aerial mycelium negative; AT⁻, non-production of aureothricin; Arg⁻, requirement for arginine for growth; Mal⁻, non-utilization of maltose; His⁻, requirement for histidine for growth.

‡ (m) denotes a minor species.

§ AF, acriflavine; NTG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine.

Fig. 1. Agarose gel electrophoresis of restriction endonuclease digests of pSK3*, pSK1* and pSK2*. 1, pSK1*/*Bgl*II; 2, pSK2*/*Bgl*II; 3, pSK3*/*Bgl*II; 4, pSK1*/*Bam*HI; 5, pSK2*/*Bam*HI; 6, pSK3*/*Bam*HI; 7, pSK1*/*Bam*HI + *Bgl*II; 8, pSK2*/*Bam*HI + *Bgl*II; 9, pSK3*/*Bam*HI + *Bgl*II; 10, λ DNA/*Hind*III; 11, pSK1*/*Eco*RI; 12, pSK2*/*Eco*RI; 13, pSK3*/*Eco*RI; 14, pSK1*/*Eco*RI + *Bgl*II; 15, pSK2*/*Eco*RI + *Bgl*II; 16, pSK3*/*Eco*RI + *Bgl*II; 17, λ DNA/*Hind*III.

Restriction endonuclease cleavage map of pSK3*

The restriction endonuclease cleavage pattern of pSK3* DNA isolated from strain A1R6(pSK3*) and pSK3 DNA isolated from strain 18a was compared. Single digestion of pSK3* with *Bgl*II, *Eco*RI, *Kpn*I or *Sac*I provided fragments identical with those of pSK3 (data not shown). Further analysis of single digestions with *Bcl*I, *Bam*HI and *Sal*I and double digests with *Bgl*II or *Eco*RI and another restriction endonuclease also provided fragments identical with those of pSK3 (data not shown).

Complete digestion of pSK3* with *Bgl*II yielded four fragments, the largest, 10.9 kb, identical with the *Bgl*II fragment of pSK2* isolated from strain R6N2(pSK2*). The others (6.8, 3.3 and 1.0 kb) appeared identical with those of *Bgl*II fragments of pSK1* isolated from strain R6N2(pSK1*) (Fig. 1). Furthermore, double digests of pSK3*, pSK1* and pSK2* with *Bgl*II and another restriction endonuclease yielded fragments from pSK3* which were assignable to those from pSK1* and pSK2*. For example, in the double digestions with *Bgl*II/*Bam*HI, and *Bgl*II/*Eco*RI, pSK3* yielded seven and six fragments, respectively, assignable to those obtained from pSK1* and pSK2*. However, single digestion of pSK3* with *Bam*HI or *Eco*RI yielded two fragments distinct from those obtained from pSK1* and pSK2* (Fig. 1). Based on these results, and data obtained with other restriction endonucleases, a restriction endonuclease cleavage map of pSK3* was constructed (Fig. 2). It became obvious that pSK3* was a cointegrate plasmid of pSK1* and pSK2*, as shown later. Since in the single digestion with different restriction endonucleases only *Bgl*II provided fragments identical in size with those from pSK1* and pSK2* (Fig. 1), it appeared that pSK1* and pSK2* were joined close to their *Bgl*II sites.

Pock-forming ability of pSK3* and its resistance to pock formation by pSK1* and pSK2*

The restriction pattern of pSK3* suggested that this plasmid might possess two different pock-forming determinants and two different resistance determinants against pock formation

Fig. 2. Restriction endonuclease cleavage map of pSK3*.

Fig. 3. Pocks induced by strain A1R6 containing pSK3* on lawns of strain A1R6 containing (a) no plasmid, (b) pSK1* and (c) pSK2*.

equivalent to those of pSK1* and pSK2*. To answer these questions, pSK3* and deletion derivatives obtained from it were characterized in terms of pock formation and pock resistance. Strain A1R6 carrying pSK1*, or pSK2* or no plasmid was challenged with A1R6 containing pSK3*, and vice versa. Strain A1R6(pSK3*) induced pocks on lawns not only of plasmid-free strain A1R6, but also of strains A1R6(pSK1*) and A1R6(pSK2*) (Fig. 3). However, it induced no pocks on lawns of strain A1R6(pSK3*). On the other hand, strains A1R6(pSK1*), A1R6(pSK2*) and A1R6(pSK1*/pSK2*) did not induce pocks on lawns of strain A1R6(pSK3*), whereas strain A1R6(pSK1*) induced pocks on lawns of strain A1R6(pSK2*),

Table 2. *Pock-forming ability of pSK3* and its derivatives and their resistance to pock formation by pSK1* and pSK2**

Plasmid(s) contained by the strain tested	Pock formation† on lawns of the strain containing				Resistance to pock formation‡ by	
	No plasmid	pSK1*	pSK2*	pSK3*	pSK1*	pSK2*
pSK3*	+	+	+	—	R	R
pSK3*-D1	+	—	+	—	R	S
pSK3*-D2	+	+	—	—	S	R
pSK3*-D3	+	+	+	—	R	R
pSK3*-D4	+	—	+	—	R	S
pSK1*	+	—	+	—	R	S
pSK2*	+	+	—	—	S	R
pSK1*/pSK2*	+	+	+	—	R	R

† +, Pocks induced; —, no pocks induced.

‡ R, resistance to pock formation by pSK1* or pSK2* (no pock formation by the strain carrying pSK1* or pSK2* on lawns of the test strain); S, sensitive to pock-formation by pSK2* or pSK1* (a strain carrying pSK2* or pSK1* induced pocks on lawns of the test strain).

and vice versa (Table 2). Thus, strain A1R6 carrying pSK3* showed the same function as that of strain A1R6 carrying both pSK1* and pSK2* in terms of pock formation and pock resistance, indicating that pSK3* carries two pock-forming determinants and two pock-resistance determinants indistinguishable from those of pSK1* and pSK2*. This conclusion was confirmed by the analysis of strains carrying deletion derivatives of pSK3*. Four deletion derivatives (pSK3*-D1–D4) of pSK3* were constructed from a *Bgl*III digest of pSK3*, which generated four fragments: A (10.9 kb), B (6.8 kb), C (3.3 kb) and D (1.0 kb) (Fig. 4). Strain R6N2 containing pSK3*-D1 (11.1 kb) or pSK3*-D4 (10.1 kb), both of which lacked the A fragment corresponding to the pSK2* part of pSK3*, was indistinguishable from the pSK1*-containing strain in pock-forming ability and pock resistance (Table 2). In contrast, strain R6N2 carrying pSK3*-D2 (10.9 kb), which lacked fragments B, C and D (i.e. the pSK1* domain of pSK3*), was indistinguishable from this strain carrying pSK2* in pock-forming ability and pock resistance. Strain R6N2 carrying pSK3*-D3 (21.0 kb), which lacked the 1.0 kb D fragment of pSK1* and pSK3*, exhibited the same pock-forming ability and pock resistance as this strain carrying pSK3*.

Plasmid transfer in pock formation

To investigate plasmid transfer from strains containing pSK3* to a plasmid-free strain in pock formation, viable hyphae (200–300) of a diluted culture (0.1 ml) of strain R6N2(pSK3*), obtained by transformation of R6N2 with pSK3* DNA, were plated on a GMY plate together with a large excess of hyphae (5×10^8) of a culture (0.2 ml) of plasmid-free R6N1 to induce pocks on the lawn of the latter strain. Spores or mycelium from pocks were picked with a tooth pick and suspended in a small volume of water. These suspensions were spread on a GMY plate and incubated. Well-grown colonies were replica plated on diagnostic plates. Almost all the colonies (1000–2000 per pock) obtained from the two pocks examined had the Mal[–] phenotype identical with that of the strain of the lawn (R6N1). Twelve colonies derived from each pock (selected at random) were examined for plasmid content. Of these 24 colonies, 19 harboured only pSK3* and the remaining five contained pSK3* and one or two species of plasmids corresponding to pSK1* and/or pSK2* (their contents varied depending on the colonies tested) (Fig. 5). These species of plasmids proved identical with pSK1* or pSK2* in their restriction endonuclease cleavage sites, pock-forming ability and pock resistance.

Since the strain bearing pSK3* (a cointegrate of pSK1* and pSK2*) showed the same pock formation behaviour as that of the strain containing both pSK1* and pSK2*, it was questionable if its pock formation on lawns of strains carrying pSK1* or pSK2* resulted from transfer of

Fig. 4. Linearized illustration of pSK1*, pSK2* and pSK3* together with deletion derivatives of pSK3*. Bgl/II digestion of pSK3* yields four fragments, A, B, C and D.

Fig. 5. Agarose gel electrophoresis of plasmids of Mal⁻ colonies derived from pocks which were induced by R6N2(pSK3*) on a lawn of R6N1 carrying no plasmid (a), pSK1* (b) or pSK2* (c). 1, pSK3* alone [19 out of 24 colonies from (a)]; 2, pSK3* and pSK1*/pSK2* [5 out of 24 colonies from (a)]; 3, pSK3* and pSK1* [22 out of 24 colonies from (b)]; 4, pSK3* and pSK2* [21 out of 24 colonies from (c)]; 5, pSK1* and pSK2* [5 out of 48 colonies from (b) and (c)]; 6, strain R6N1(pSK1*); 7, strain R6N1(pSK2*); 8, strain R6N2(pSK3*-D4).

pSK3* itself or of pSK1* and pSK2* segregated from pSK3*. To answer this question, two kinds of pocks caused by strain R6N2(pSK3*) on lawns of strains R6N1(pSK1*) and R6N1(pSK2*) were also examined. All the colonies (1000–2000 colonies per pock) from two pocks on each lawn had the Mal⁻ phenotype of R6N1. When 12 colonies derived from each pock (selected at random) were examined for their plasmids, the most common class was confirmed by agarose gel electrophoresis to harbour pSK3* together with the plasmid corresponding to pSK1* or pSK2* carried by the strain of the lawn. Some of them (2–4 colonies) seemed to contain both plasmids corresponding to pSK1* and SK2* in addition to pSK3*, indicative of segregation. The rest (1–2 colonies) did not contain pSK3* but two species of plasmids

corresponding to pSK1* or pSK2* (Fig. 5), and induced pocks on lawns of strains carrying pSK1* or pSK2*. When pocks produced by these strains on a lawn of a plasmid-free strain were examined for their plasmid content, strains carrying either one of these plasmids were found, and these plasmids proved identical with pSK1* or pSK2* in restriction endonuclease cleavage patterns, pock-forming ability and pock resistance.

Plasmid transfer of pSK3*-D3 in pock formation by strain R6N2(pSK3*-D3) was also examined in the same way as described above. Among a total of 120 *Mal*⁻ colonies from 10 pocks produced on a lawn of plasmid-free strain R6N1, 114 colonies contained pSK3*-D3. However, 19 colonies contained one or two species of plasmids corresponding to pSK2* or pSK3*-D4 in addition to pSK3*-D3, indicating segregation. The remaining six colonies did not contain pSK3*-D3, but one or two species of plasmids corresponding to pSK2* or pSK3*-D4. The transfer of pSK3*-D3 and the occurrence of two species of plasmids corresponding to pSK2* or pSK3*-D4 were also observed in pock formation on lawns of strains carrying pSK1* or pSK2* (data not shown).

DISCUSSION

A few strains carrying only pSK3 with high pock-forming ability (pSK3*) were obtained from *S. kasugaensis* MB273 derivatives 18a and S1A1, which harbour three plasmids, pSK1, pSK2 and pSK3. Restriction endonuclease cleavage of pSK3* yielded fragments which were indistinguishable from those of pSK3 isolated from strain 18a and were assignable to those from pSK1* and pSK2*. Furthermore, characterization of pock formation and pock resistance of pSK3* and its deletion derivatives revealed that pSK3* contained two pock-forming determinants and two pock-resistance determinants corresponding to those of pSK1* and pSK2*. Therefore, it was concluded that pSK3* was a composite plasmid consisting of two elements (pSK1* and pSK2*) joined together near their *Bgl*II sites. The occurrence of pock-forming variants of natural plasmids has been reported for SCP2 in *S. coelicolor* A3(2) (SCP2*; Bibb & Hopwood, 1981) and for pSK1* and pSK2* from pSK1 and pSK2 in *S. kasugaensis* MB273 (Akagawa *et al.*, 1984). To our knowledge, pSK3* represents the first report of a plasmid species possessing two replication determinants on the same replicon in the actinomycetes. Many drug-resistance plasmids (R plasmids) of *Enterobacteriaceae* (Perlman & Rownd, 1976; Danbara *et al.*, 1980) and thermophilic bacilli (Imanaka *et al.*, 1984) are composite replicons. In this context, pSK3* is unique since no resistance determinants have been assigned to pSK1 and pSK2 of *S. kasugaensis* MB273.

Strains carrying pSK3* or pSK3*-D3 (which lacked the smallest *Bgl*II fragment in the pSK1* part of pSK3*) were identical with the strain carrying both pSK1* and pSK2* in terms of pock formation and pock resistance. This indicates that the *Bgl*II fragment is not essential for the expression of two pock-forming determinants and pock-resistance determinants. This is consistent with the observation that elimination of the smallest *Bgl*II fragment from pSK1* resulted in no change in pock formation with pSK1* (Akagawa *et al.*, 1984).

It has been reported that pock formation by SCP2* in *S. coelicolor* A3(2) (Bibb & Hopwood, 1981), by pIJ101 in *S. lividans* (Kieser *et al.*, 1982) and by pSK1* and pSK2* in *S. kasugaensis* MB273 (Akagawa *et al.*, 1984) was associated with plasmid transfer from plasmid-containing cells to plasmid-free cells. In this report the transfer of pSK3* and pSK3*-D3 from their carrier cells to recipient mycelial hyphae of the lawn was also demonstrated in pock zones on lawns of strains carrying pSK1*, pSK2* or no plasmid. Therefore, this cointegrate maintains two functional transfer and pock functions. It can form pocks on strains which contain one of the plasmids which form part of the cointegrate. By contrast, strains carrying pSK1* or pSK2* individually are resistant to the pock-forming ability of other strains carrying the respective plasmids, but are not cross-resistant (Table 2). Strains containing the cointegrate are, however, resistant to pock formation induced by strains containing the individual components of the cointegrate. Therefore, the pock resistance is quite different from plasmid incompatibility in, for example, *Escherichia coli* (Clowes, 1972).

Segregation of deletion derivative plasmids of pSK3* and pSK3*-D3 was observed at frequencies of about 20–25% of pock-forming units. Thus, 20–25% of pocks derived from pSK3* yielded pSK1* and pSK2* (Fig. 5); pSK3*-D3 yielded pSK3*-D4 and pSK2* (data not shown). The same segregation was obtained by subculturing strains carrying pSK3* or pSK3*-D3 (data not shown). The mechanism of this specific segregation is unknown. However, it is possible that association and dissociation between pSK1* and pSK2* occur through *in vivo* recombination between the homologous DNA regions, since in preliminary DNA–DNA hybridization experiments, DNA homology was detected only between the smallest *Bgl*II fragment of pSK1* (sites 1–3 in Fig. 2) and a *Bgl*II/*Eco*RI fragment of pSK2* (sites 15–20 in Fig. 2) and between an *Eco*RI/*Bgl*II fragment of pSK1* (sites 13–15 in Fig. 2) and that of pSK2* (sites 20–1 in Fig. 2) (data not shown). The mechanisms of plasmid replication and plasmid-mediated conjugation are of common interest in *Streptomyces* plasmids. The pock-forming composite plasmid pSK3* in *S. kasugaensis* MB273 may be useful in investigating these functions.

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REFERENCES

- AKAGAWA, H., KAWAGUCHI, K. & ICHIHARA, M. (1984). Plasmids of *Streptomyces kasugaensis* MB273: their pock formation, their dispensable endonuclease cleavage sites for pock formation, and transformation of *S. kasugaensis* MB273 by them. *Journal of Antibiotics* **37**, 1016–1025.
- BIBB, M. J. & HOPWOOD, D. A. (1981). Genetic studies of the fertility plasmid SCP2 and its SCP2* variants in *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **126**, 427–442.
- BIBB, M. J., FREEMAN, R. F. & HOPWOOD, D. A. (1977). Physical and genetical characterization of a second sex factor, SCP2, for *Streptomyces coelicolor* A3(2). *Molecular and General Genetics* **154**, 155–166.
- BIBB, M. J., SCHOTTEL, J. L. & COHEN, S. N. (1980). A DNA cloning system for interspecies gene transfer in antibiotic-producing *Streptomyces*. *Nature, London* **284**, 526–531.
- BIBB, M. J., WARD, J. M., KIESER, T., COHEN, S. N. & HOPWOOD, D. A. (1981). Excision of chromosomal DNA sequences from *Streptomyces coelicolor* forms a novel family of plasmids detectable in *Streptomyces lividans*. *Molecular and General Genetics* **184**, 230–240.
- CLOWES, R. C. (1972). Molecular structure of bacterial plasmids. *Bacteriological Reviews* **36**, 361–405.
- DANBARA, H., TIMMIS, J. K., LURZ, R. & TIMMIS, K. N. (1980). Plasmid replication functions: two distinct segments of plasmid R1, RepA and RepD, express incompatibility and are capable of autonomous replication. *Journal of Bacteriology* **144**, 1126–1138.
- FURUMAI, T., TAKEDA, K. & OKANISHI, M. (1982). Function of plasmids and alteration of phenotypes caused by protoplast regeneration in *Streptomyces kasugaensis*. *Journal of Antibiotics* **35**, 1367–1373.
- HOPWOOD, D. A., HINTERMANN, G., KIESER, T. & WRIGHT, H. M. (1984). Integrated DNA sequences in three streptomycetes form related autonomous plasmids after transfer to *Streptomyces lividans*. *Plasmid* **11**, 1–16.
- IMANAKA, T., ANO, T., FUJI, M. & AIBA, S. (1984). Two replication determinants of an antibiotic-resistance plasmid, pTB19, from a thermophilic bacillus. *Journal of General Microbiology* **130**, 1399–1408.
- KIESER, T., HOPWOOD, D. A., WRIGHT, H. M. & THOMPSON, C. J. (1982). pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. *Molecular and General Genetics* **185**, 223–238.
- LYDIATE, D. J., MALPARTIDA, F. & HOPWOOD, D. A. (1985). The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. *Gene* **35**, 223–235.
- PERLMAN, D. & ROWND, R. H. (1976). Two origins of replication in composite R plasmid DNA. *Nature, London* **259**, 281–284.
- SHIRAHAMA, T., FURUMAI, T. & OKANISHI, M. (1981). A modified regeneration method for *Streptomyces* protoplasts. *Agricultural and Biological Chemistry* **45**, 1271–1273.
- THOMPSON, C. J., WARD, J. M. & HOPWOOD, D. A. (1980). DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. *Nature, London* **286**, 525–527.