POCK-FORMING PLASMIDS ISOLATED FROM *STREPTOMYCES ROSEOCHROMOGENUS*

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A series of high copy number plasmids designated pSRC were isolated from *Streptomyces roseochromogenus* S264. The pSRC series were found to be self-transmissible by conjugation and to elicit lethal zygosis (Ltz). Using the Ltz phenotype to detect plasmid transformants, the pSRC plasmids were shown to have a wide host range. Among them pSRC1 consisted of two different plasmids with the same molecular weight, pSRC1a and 1b. Information regarding restriction sites suitable for the insertion of DNA was obtained by cloning the thiostrepton resistance gene from pIJ702 into pSRC1b. The single *Bgl* II site of pSRC1b was nonessential for replication and pock-formation. The pSRC plasmids may be suitable as cloning vectors in *Streptomyces*.

In previous papers[1,2] we have described a plasmid pSL1 isolated from *Streptomyces lavendulae* S985. Though this plasmid had many advantages as a cloning vector such as a high copy number, a low molecular weight and suitable cloning sites for foreign DNA, it lacked selective markers. Recently gene cloning systems have been established in *Streptomyces* using plasmids which generated 'pocks' (circular zones of sporulation inhibition associated with plasmid transfer)[3~6]. These pock-generating plasmids were SCP2, SCP2*[7], the SLP1 family[3] and pIJ101 series[6], which were isolated and developed by HOPWOOD and co-workers. The pIJ101 series are especially useful vectors because of a broad host range and a high copy number. Here we describe the pSRC series which could be developed into alternative host-vector systems for *Streptomyces* strains.

Materials and Methods

Strains

S. lavendulae S985 (KCC-S0985) and *S. roseochromogenus* S264 (KCC-S0264) were kindly provided by Dr. A. SEINO, Kaken Chemical Co. *S. lividans* JI1326, JI3131 and *S. parvulus* 2283 obtained from Prof. D. A. HOPWOOD, John Innes Institute. Plasmid pSRC series and pIJ702 were isolated from S264 and JI3131, respectively.

Isolation of Plasmid DNA

S. roseochromogenus S264 was grown in liquid TSBG medium consisting of Tryptic Soy Broth (Difco) supplemented with 1% glucose and 0.1% glycine. Plasmid DNA was isolated using a method of alkaline denaturation[10]. Further purification of each plasmid of pSRC series was as follows. After electrophoresis of pSRC plasmids in 0.7% agarose gels, each plasmid band was cut out with a razor blade and the DNA was extracted by the electroelution method[11]. Ethidium bromide was removed from the DNA solution with phenol followed by chloroform, and the plasmid DNA was precipitated with ethanol before transformation and restriction enzyme analysis.

Protoplast Transformation with Plasmid DNA

Protoplasts were generated by the method of OKANISHI *et al.*[12] and the protoplast transformation procedure was according to THOMPSON *et al.*[6].

Cleavage of Plasmids with Restriction Endonucleases

Restriction endonucleases, *Bcl* I, *Sst* I and *Sst* II were purchased from BRL Inc. and other restriction endonucleases from Takara Shuzo Co. Cleavage of plasmid DNA was according to the manufacturers' recommendation. Agarose gel electrophoresis was performed in 40 mM Tris - 20 mM sodium acetate - 2 mM EDTA, adjusted to pH 7.7 with acetic acid.

Introduction of Thiostrepton Resistant Gene into pSRC1

pIJ702, originated and developed from pIJ101, carries thiostrepton resistant (*tsr*) and tyrosinase genes[4]. As the thiostrepton resistant gene was isolated from pIJ702 by cleavage with *Bcl* I, the introduction of *tsr* gene into pSRC1 was as follows: pSRC1 and pIJ702 were digested with *Bgl* II and *Bcl* I, respectively and ligated with T4 ligase at 4°C overnight. The ligated mixture was transformed into *S. lividans* 1326 protoplasts. After regeneration on R2YE medium[5], the plates were overlayed with 2.5 ml soft agar (Nutrient Broth (Difco) supplemented with 0.5% agar) containing 500 μg/ml thiostrepton.

Measurement of Plasmid Copy Number

Plasmid copy number was determined by the method of KIESER *et al.*[9].

Results

Isolation of pSRC Plasmids

S. roseochromogenus S264 was found to have four plasmids (Fig. 1); which were designated pSRC1, 2, 3 and 4 in order of increasing size. The sizes of plasmids were 10 kb (pSRC1), 23 kb (pSRC2), 32 kb (pSRC3) and above 40 kb (pSRC4).

Transfer of Plasmids to Other *Streptomyces* Species

When S264 was replica-plated onto a lawn of *S. lividans* 1326, S264 was surrounded by a narrow zone of sporulation inhibition. Such a zone is known to be due to the presence of conjugative plasmids[18]. In order to confirm the Ltz phenotype and presence of pSRC plasmids, *S. lividans* 1326 was transformed with pSRC plasmids mixture. Regenerated protoplasts of 1326 readily produced 'pocks' (Fig. 2) and all of the pSRC plasmids were isolated from the 'pocks'. These results indicated that pSRC plasmids had a pock-forming ability. To determine host range of pSRC plasmids, the plasmids mixture was transformed into *S. lavendulae* S985, *S. parvulus* 2283 and *S. actuosus* ATCC 25421. All of the strains showed 'pocks' after transformation like *S. lividans* 1326 and all of the pSRC plasmids were isolated from the 'pocks'. This result suggested a wide host range for the plasmids.

Fig. 1. Agarose gel electrophoresis of pSRC plasmids.

1. λDNA digested with *Hin*d III, 2. pSRC plasmids.

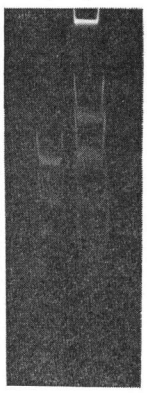
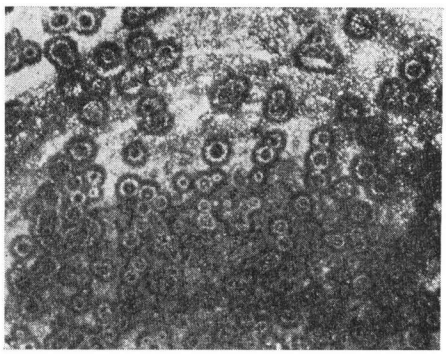


Fig. 2. 'Pocks' produced by pSRC on a plasmid-free lawn of *S. lividans* 1326.



pSRC0

The pSRC0, purified as described in Materials and Methods was transformed into *S. lividans* 1326. From one of the 'pocks' after transformation with pSRC1, a new plasmid designated pSRC0 was isolated. Two possible explanations were suggested: conversion of pSRC1 to pSRC0 in the transformant, or contamination by pSRC0 in the pSRC1 preparation. The first possibility was excluded because restriction enzyme maps of the two plasmids showed no significant similarities (see Fig. 3 and Fig. 5) and also pSRC0 revealed no homology with pSRC1 by Southern hybridization (data not shown). The second explanation was more probable. When pSRC1 was removed from gels, it might be contaminated with pSRC0 because the pSRC0 (8.85 kb) and pSRC1 (10.2 kb) migrated close in agarose gels. After transformation with the mixed plasmids, a transformant harboring pSRC0 could be isolated. A restriction enzyme map of pSRC0 is shown in Fig. 3.

Fig. 3. Restriction endonuclease map of pSRC0. The map is calibrated in kilobase pairs.

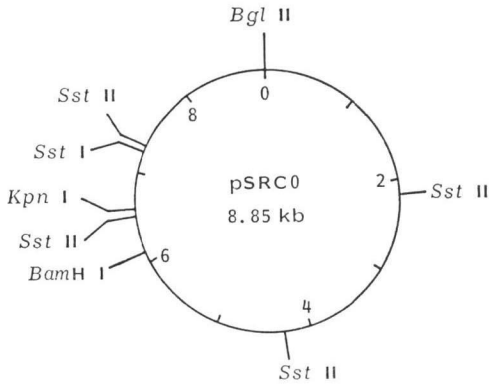


Fig. 4. Agarose gel electrophoresis of pSRC1 and pSRC1a plasmids digested with restriction endonucleases.

pSRC1 plasmid was isolated from *S. roseochromogenus* S264 after purification by agarose gel electrophoresis. pSRC1a was isolated from *S. actuosus* after transformation with pSRC1 plasmids.

A, pSRC1 plasmids digested with *Kpn* I (2), *Kpn* I and *Sst* I (3), *Bgl* II and *Sst* I (4) and *Bgl* II and *Kpn* I (5).

B, pSRC1a plasmid digested with *Kpn* I (2), *Kpn* I and *Sst* I (3), *Bgl* II and *Sst* I (4) and *Bgl* II and *Kpn* I (5).

Molecular weight markers used were PM2 DNA digested with *Hind* III (1) and λDNA digested with *Hind* III (6).

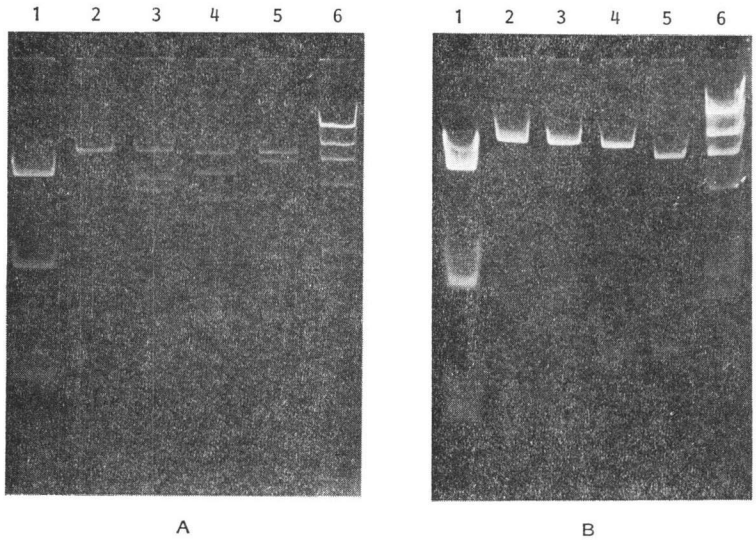


Fig. 5. Restriction endonuclease maps of pSRC1a and 1b.
The maps are calibrated in kilobase pairs.

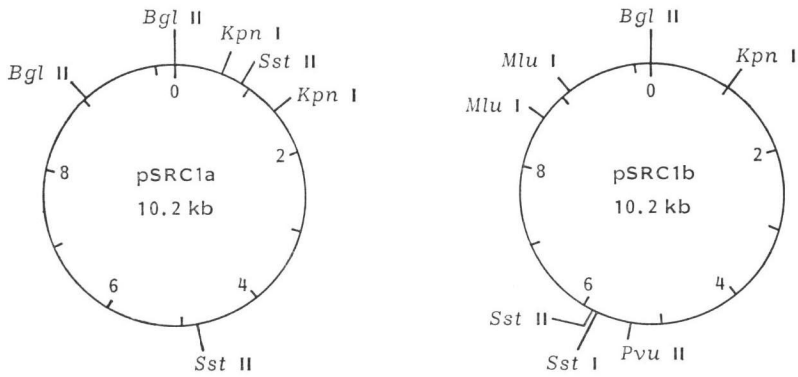
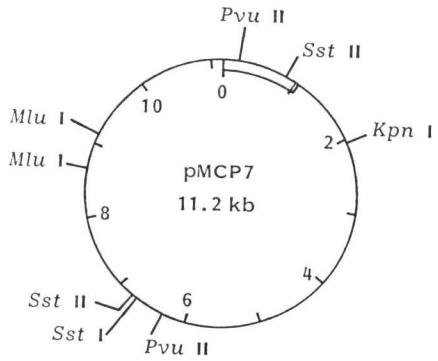


Table 1. Analysis of fragments of pSRC1 and pSRC1a plasmids digested with various restriction endonucleases.

Restriction endonuclease	Size of fragments (kilobases)	
	pSRC1	pSRC1a
Sst I	10.0~10.4	
	No cleavage	No cleavage
Kpn I	10.0~10.4	
	9.3	9.4
	0.8	0.8
Bgl II	10.0~10.4	
	8.7	8.7
	1.2	1.2
Bgl II+*Kpn* I	9.2~9.8	
	7.6~7.9	7.6~7.9
	1.2	1.2
	1.0	
	0.8	0.8
	0.6	0.6
Bgl II+*Sst* I	9.0~9.5	9.0~9.5
	6.0	
	4.2	
	1.2	1.2
Kpn I+*Sst* I	9.4~10.0	9.4~10.0
	5.5	
	4.8	
	0.8	0.8
Bgl II+*Sst* I+*Kpn* I	7.1~7.5	7.1~7.5
	4.4	
	4.0	
	1.2	1.2
	1.0	
	0.8	0.8
	0.6	0.6

Fig. 6. Restriction endonuclease map of recombinant plasmid pMCP7.

pMCP7 is pSRC1b inserted the thiostrepton resistant gene fragment from pIJ702 at *Bgl* II cleavage site. Double line indicates the thiostrepton resistant gene. The map is calibrated in kilobase pairs.



pSRC1

After purification of pSRC1 as described in Materials and Methods, a restriction enzyme cleavage analysis of pSRC1 was carried out. The results are shown in Fig. 4 and summarized in Table 1 (left column). For example, digestion of pSRC1 with *Kpn* I gave three fragments of 10.1, 9.3 and 0.8 kb. Considering the size of pSRC1 (10.2 kb), this result suggested either partial digestion or the presence of two different plasmids with the same molecular weight. As cleavage with other restriction enzymes showed similar patterns and increasing amount of enzymes had no effect on the cleavage patterns, the

latter speculation seemed probable. We postulated two molecules in the pSRC1 preparation: one with no cleavage site for *Sst* I was named pSRC1a and the other with one *Sst* I site, pSRC1b. To confirm this possibility, we tried to separate and purify each of the pSRC1 plasmids. One of the 'pocks' after transformation of *S. actuosus* with a pSRC1 preparation was shown to contain only pSRC1a (Fig. 4 and Table 1, right column). A cleavage map of pSRC1a was thus determined (Fig. 5). It revealed that what remains after removal of the cleavage fragments of pSRC1a from pSRC1 fragments (Table 1) comes from pSRC1b. Consequently, the cleavage map of pSRC1b was determined as shown in Fig. 5. From the results of cleavage patterns in gel electrophoresis, the copy number of pSRC1b was shown to be higher than that of pSRC1a in S264 cells.

To detect a dispensable region and add another selective marker for improvement of pSRC1 as a cloning vector, the thiostrepton resistant (*tsr*) gene from pIJ702 was introduced into pSRC1 as described in Materials and Methods. A plasmid designated pMCP7 was isolated from thiostrepton resistant clones. An analysis of restriction enzyme cleavage patterns of pMCP7 DNA showed that it was a recombinant plasmid between pSRC1b and the thiostrepton resistant gene. A cleavage map of pMCP7 was determined (Fig. 6). This map of pMCP7 supported the restriction enzyme map of pSRC1b shown in Fig. 5. By retransformation with pMCP7 into *S. lividans* 1326, pMCP7 was shown to elicit 'pocks' and give *tsr* clones. These results showed that the *Bgl* II site of pSRC1b is located in a region nonessential for replication and 'pock' formation.

Copy Number

Copy numbers of pSRC0 and pSRC1 (pSRC1a and 1b) were determined in transformants. Assuming 10^4 kb for the size of chromosomal DNA, the copy number of pSRC0 was estimated about 150 copies per chromosome and that of pSRC1 was a little lower.

Discussion

Among a newly isolated group of plasmids pSRC, several (pSRC0, pSRC1a and pSRC1b) were further examined. They have useful properties as cloning vectors in *Streptomyces*, that is, pock formation, high copy number, wide host range, though it remains to be known whether pSRC1b can be transformed into *S. actuosus*. pSRC1b has a single *Bgl* II cloning site in a region nonessential for replication and pock formation. Since *Bgl* II cleavage generates cohesive ends identical with those obtained by *Bam*H I, *Bcl* I and *Mbo* I digestion, the *Bgl* II site of pSRC1b is available for cloning of foreign DNA digested with these four enzymes. pMCP7 is also a useful vector because the *Cla* I site within the *tsr* fragment could be used for insertional inactivation[14].

KIESER *et al.* have isolated similar group of plasmids, which are multicopy, broad host range and pock-forming[9]. Three smaller plasmids among them appeared to be naturally occuring deletion variants of the largest one. It is unknown whether the pSRC plasmids are related; as pSRC2, 3 and 4 were relatively large, reliable restriction enzyme maps could not be determined.

Addendum in Proof

It has been found that the restriction enzyme map of pSRC0, insofar as the same endonucleases were studied, appears to be almost identical with that of pIJ101, except that pSRC0 lacks the single *Pst* I site which exists in pIJ101.

Acknowledgments

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