CONSTRUCTION OF PLASMID VECTORS FROM *STREPTOMYCES KASUGAENSIS* PLASMIDS, pSK1 AND pSK2

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Streptomyces kasugaensis G3 was transformed by pIJ702 DNA carrying the thiostrepton-resistance gene at a frequency of 2×10^5 transformants/μg DNA, but it was found that the introduced pIJ702 was very unstable in this strain. This result led us to make useful vectors using the stable plasmids resident in *S. kasugaensis*. The *Bcl* I-fragment, containing the thiostrepton-resistance gene obtained from pIJ702, was inserted into the pSK1 and pSK2 plasmids isolated from *S. kasugaensis*. Two composite plasmids, pSK11-1 (8.0 Md) and pSK21-1 (4.8 Md), were isolated from the thiostrepton-resistant transformants of strain G3. The constructed pSK11-1 consisted of the entire pSK1 molecule and the thiostrepton-resistance gene fragment. pSK21-1 consisted of the large *Bcl* I-fragment of pSK2 (4.1 Md) and the same thiostrepton-resistance gene. These plasmids were stably maintained in *S. kasugaensis* G3. Small derivatives of these composite plasmids were prepared by restriction enzyme cleavage and self-ligation, and several unique insertion sites were also constructed in these small plasmids. By analysis of the physical maps of these plasmids, the essential regions of pSK1 and pSK2 were determined from their DNA segments to be 2.5 Md in pSK11-1 and 1.9 Md in pSK21-1. pSK21-B5, one of these plasmid vectors, showed a wide host range in the genus *Streptomyces* and was stably maintained in all streptomycete species tested, except *S. kasugaensis* M338.

Streptomyces species are Gram-positive mycelial bacteria with high morphological differentiation, and produce a variety of important antibiotics of medical and agricultural use and other biologically active substances. The application of gene cloning techniques to streptomycetes is expected to be useful in solving many scientifically or technically difficult problems. Some examples are: a rational procedure for increasing antibiotic productivity in fermentation; introduction of new enzymatic steps to antibiotic synthetic pathways for the creation of new antibiotics; understanding the mechanisms and genetic regulation of the resistance of a producer to its own antibiotic; analysis of streptomycete morphological differentiation or development from the point of view of the regulation of gene expression.

With these objectives, scientists have performed extensive studies to establish the following gene cloning systems: method of protoplast regeneration[1,2], utilization of pock-forming plasmids[3], development of a transformation method for protoplasts by plasmids[3,4], and liposome-mediated transformation[5]. The studies on these fundamental techniques have resulted in the construction of useful plasmid vectors carrying antibiotic resistance genes or some other genes as selective markers[6~10]. Recently, several antibiotic biosynthetic genes[11,12] and a pleiotropic gene controlling the production of A-factor and two pigments[13] were cloned in *Streptomyces*. Almost all of the cloning experiments covering the technical problems described above, however, have been limited to *Streptomyces coelicolor* A3(2) or *Streptomyces lividans* as the host strain. In practical terms, it will be necessary to develop cloning systems in many other streptomycete strains corresponding to the antibiotic to be produced

or studied.

Streptomyces kasugaensis MB273 produces aureothricin and kasugamycin, and it carries two small multi-copy plasmids, pSK1 and pSK2[14]. With the objective of developing a cloning system for *S. kasugaensis*, we have already solved several technical problems encountered and have examined the conditions required for using it as a host[15]. As the next step, useful plasmid vectors for *S. kasugaensis* need to be constructed.

In the research covered by this paper, first the application of pIJ702 as a cloning vector in *S. kasugaensis* G3 was tested from the point of view of its stability in the strain. Thereafter, we successively investigated insertion of the thiostrepton-resistance gene (*tsr*) of pIJ702 into pSK1 and pSK2, the construction of small plasmid vectors, preparation of plasmids carrying multi-insertion sites, the host range in various species of the genus *Streptomyces*, and the stability of the plasmids in the transformed species.

Materials and Methods

Bacterial Strains and Plasmids

S. kasugaensis strains 189, R5, and G3, derivatives previously isolated from *S. kasugaensis* MB273, were used. The pSK1 and pSK2 plasmids were extracted from strains 189 and R5, respectively. *S. kasugaensis* G3 was used as the host strain of the plasmids to be tested. *S. lividans* 3131, provided by KATZ, and *S. lividans* TK21, provided by HOPWOOD, were used for extraction of pIJ702 and as the host for these plasmids, respectively.

Media, Reagents and Enzymes

GPY medium consisted of glucose 1.0%, Polypepton 0.4%, yeast extract 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.1%, pH 7.0. GPYG medium[15] and R3 medium[2] for preparation and regeneration of protoplasts, respectively, have been described previously. Instead of thiostrepton, thiopeptin obtained from Fujisawa Pharmaceutical Co., Ltd. was used for the selection of thiostrepton-resistant transformants and to test the stability of the plasmids. Restriction endonucleases, bacteriophage T4 DNA ligase and calf intestine alkaline phosphatase were purchased from New England Biolabs, Inc., Boehringer-Mannheim Yamanouchi Co., Ltd., or Wako Chemical Co., Ltd.

Preparation of Plasmid DNAs, Digestion with Restriction Enzymes, and Agarose Gel Electrophoresis

All plasmids were prepared by the cleared lysate method as described previously[15]. The methods for restriction enzyme digestion and agarose gel electrophoresis were virtually identical with those described previously[15], except that the gel electrophoresis was carried out using Tris-phosphate buffer. In agarose gel electrophoresis of DNA, *Hind* III fragments of λ DNA were used as molecular weight standards.

Transformation of Protoplasts

Protoplasts were prepared from the mycelia grown in GPYG medium for 72 hours. Transformation was achieved with polyethylene glycol 2000 at 0°C. Protoplasts (2.0×10^8) in 100 μl of PWP[2], 0.1 μg of plasmid DNA diluted with PWP to make 100 μl, and 200 μl of 40% PEG 2000 were mixed and kept for 2 minutes at 0°C. The reacted mixture was diluted with 0.6 or 2.6 ml of PWP and some of 0.1 ml were put on the regeneration plates (R3), followed by embedding with the upper layer agar medium of R3. For the selection of thiostrepton-resistant transformants, R3 medium (3 ml) containing 300 μg of thiopeptin was overlaid after incubation at 28°C for 18~20 hours and the transformants were detected after incubation for 7~9 days.

Detection of Plasmids from Transformants

Plasmids in transformants were detected by a modification of the alkaline lysis method reported by CHATER *et al.*[4]. The mycelia grown on a GPY agar plate for 2~3 days were harvested and sus-

pended in 0.1 ml of lysozyme-RNase solution (8 mg of lysozyme and 1 mg of RNase per ml in 2× TES[15]). After incubation for 30 minutes at 37°C, 0.1 ml of lytic mixture[4] adjusted at 0.4 N NaOH was added to the suspension and held at room temperature for 10 minutes. Then 0.05 ml of 2.0 M Tris-HCl (pH 7.0) and 2.0 ml of phenol - $CHCl_3$ solution were added and mixed vigorously. The upper phase (10~15 μl) separated by centrifugation was submitted directly to agarose gel electrophoresis.

Insertion of Thiostrepton-resistance Gene into pSK Plasmids

The *Bcl* I fragments of pIJ702 (1.0 μg) were treated with calf intestine alkaline phosphatase[17], heated at 70°C for 30 minutes, and then mixed with the *Bcl* I fragments of pSK1 or pSK2 (1.2 μg each). Their DNA fragments were precipitated with cold EtOH and ligated with T4 DNA ligase. The ligation mixture was introduced into the protoplasts of *S. kasugaensis* G3 by the transformation method and their protoplasts were incubated on R3 medium. From the thiopeptin-resistant transformants which grew, composite plasmids were isolated and their physical maps were determined.

Construction of Small Plasmids and Introduction of Multi-insertion Sites

The composite plasmid obtained was digested with restriction enzyme, treated with phenol, and precipitated with EtOH, and the DNA fragments were re-ligated with T4 DNA ligase at 22°C for 2 hours. The ligation mixture was introduced into G3 protoplasts and thiopeptin-resistant clones were selected. Small plasmids were screened by extracting plasmids from the transformant clones and by estimating their molecular sizes on agarose gel electrophoresis. For the preparation of plasmids carrying multi-insertion sites, the desired DNA fragment was extracted by electrophoresis from the agarose gel kept in a dialysis tube. Restriction enzyme digestion, ligation and transformation procedures were carried out as described above.

Stability of Plasmids in Streptomycete Mycelia

The clones carrying thiostrepton-resistance plasmid were pre-incubated in GPY medium containing thiopeptin (10 μg/ml) for 48 hours and then transferred into GPY medium without thiopeptin by inoculation at 0.1% volume. Short rod-like mycelia in the stationary phase of growth were diluted and spread on two GPY agar plates with and without thiopeptin. The stability of the thiostrepton-resistance plasmid was expressed as the ratio of the number of colonies that grew on the two plates described above.

Results

Stability of pIJ702 in *S. kasugaensis* G3

Plasmid vector pIJ702, constructed by KATZ *et al.*[13] from pIJ350[9], is a high copy number plasmid with a wide host range and contains the *tsr* and tyrosinase genes. Thinking that pIJ702 might be useful as a vector for *S. kasugaensis*, G3 protoplasts were transformed with this plasmid vector, and its stability in this strain was examined. pIJ702 was very unstable in strain G3 as shown in Fig. 1. When the G3 clones carrying pIJ702 were shake-cultured in GPY medium without thiopeptin, the ratio of thiopeptin-resistant to sensitive colonies decreased significantly during incubation, finally being a ratio less than 10% in the stationary phase of growth. Even in the medium containing thiopeptin, the ratio decreased to 75% and the isolated thiopeptin-sensitive colonies were found not to carry pIJ702. These results led us to construct useful plasmid vectors by employing the plasmids resident in *S. kasugaensis*.

Introduction of *tsr* into pSK1 and pSK2

S. kasugaensis MB273 (parent strain) carries two small multi-copy plasmids, pSK1 and pSK2, which are known to be stably maintained in *S. kasugaensis* strains[15] and to have a copy number of more than 12 (data not shown). *Bcl* I-digested pSK1 or pSK2 was mixed with the DNA fragments

Fig. 1. Stability of pIJ702 in transformed *S. kasugaensis* G3.

Strain G3 carrying pIJ702 was pre-incubated in GPY medium containing thiopeptin (10 μg/ml) for 48 hours and then inoculated in GPY medium with and without thiopeptin. Each was inoculated onto agar plates with and without thiopeptin and the stability ratio was determined after 3 days' incubation.

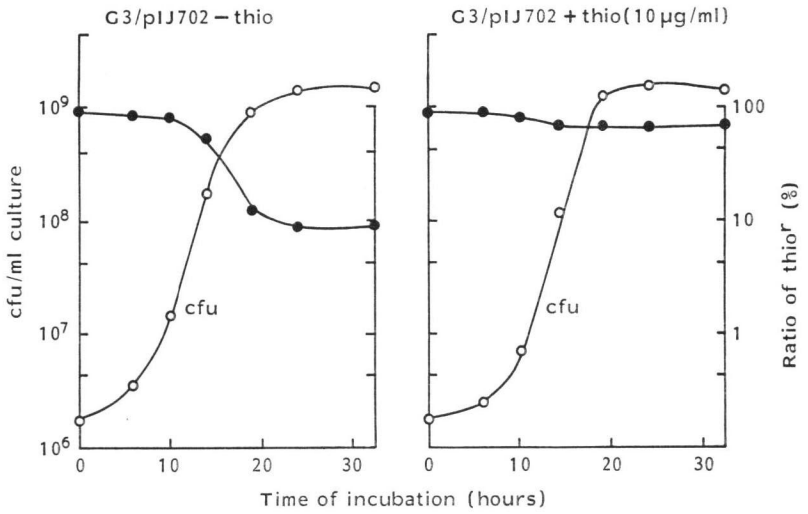
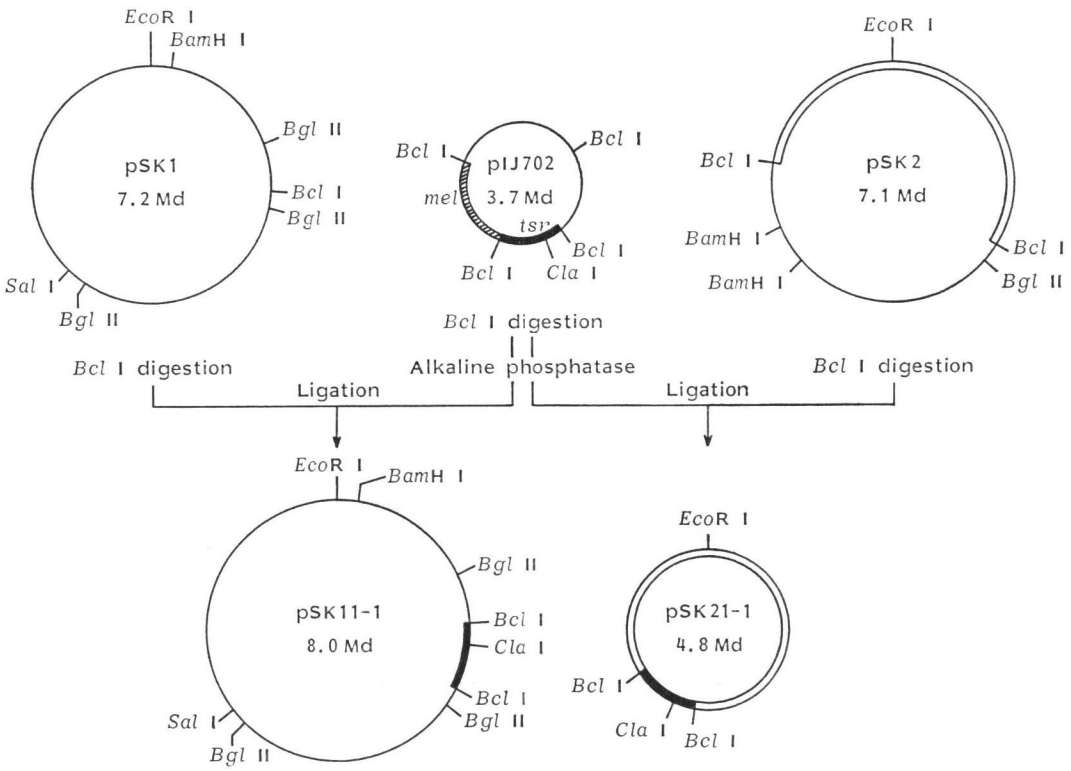


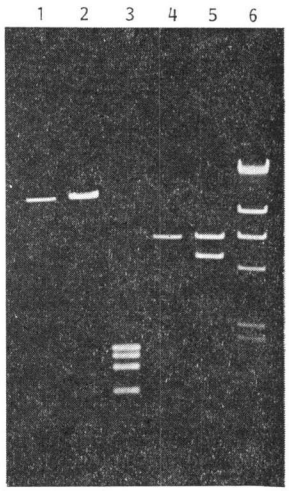
Fig. 2. Schematic procedure for construction of composite plasmids.
Some restriction sites are omitted from the plasmids.



of *Bcl* I-cleaved pIJ702 treated with calf intestine-alkaline phosphatase and then ligated (Fig. 2). Two plasmids, designated pSK11-1 and pSK21-1, were isolated as typical composite plasmids from the transformants obtained. pSK11-1 (8.0 Md) consisted of the entire pSK1 molecule plus a fragment of pIJ702 containing *tsr*, which was ligated at the *Bcl* I site (Figs. 2 and 3). pSK21-1 (4.8 Md) consisted of the larger one (4.1 Md) of two *Bcl* I-fragments of pSK2 plus the same *tsr* fragment (Figs. 2 and 3). These results indicate that the small *Bcl* I fragment of pSK2 is a dispensable region of the plasmid, and that the *Bcl* I sites of pSK1 and pSK2 are not located in the regions essential to their plasmids replication.

Fig. 3. Electrophoresis of pSK11-1 and pSK21-1 digested with *Bcl* 1.

1 pSK1; 2 pSK11-1; 3 pIJ702; 4 pSK21-1; 5 pSK2; 6 molecular weight standard.



These composite plasmids were stably maintained in strain G3 even after the strain was incubated in the liquid medium without thiopeptin (Fig. 4). The frequency of thiopeptin-resistant colonies in the culture was almost 100% at every phase during incubation. Moreover, these composite plasmids were found to be capable of introduction into *S. lividans* TK21 protoplasts and to be maintained stably therein (Table 1).

Construction of Small Plasmids from pSK11-1 and pSK21-1

In order to elucidate the essential regions of pSK1 and pSK2, small plasmids were constructed from pSK11-1 and pSK21-1 by *in vitro* deletion and self-ligation. pSK11-ΔS8 was isolated from the

Fig. 4. Stability of pSK11-1 and pSK21-1 in *S. kasugaensis* G3 transformed by them.
See the legend to Fig. 1 for procedure.

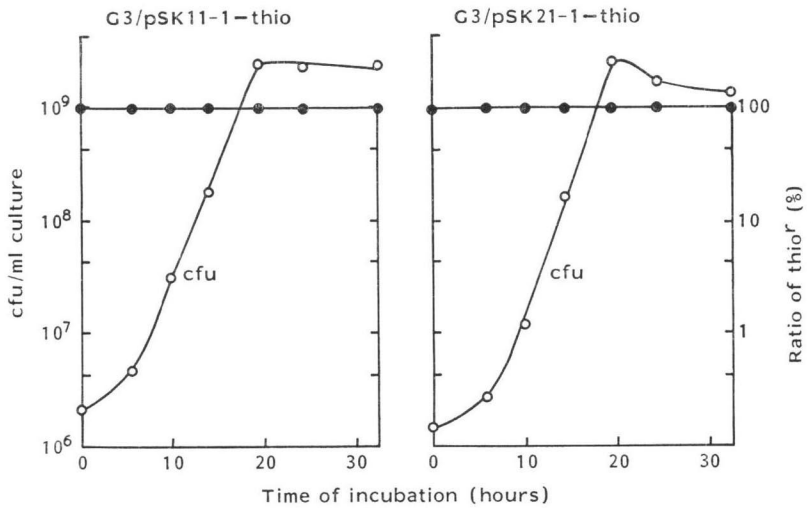
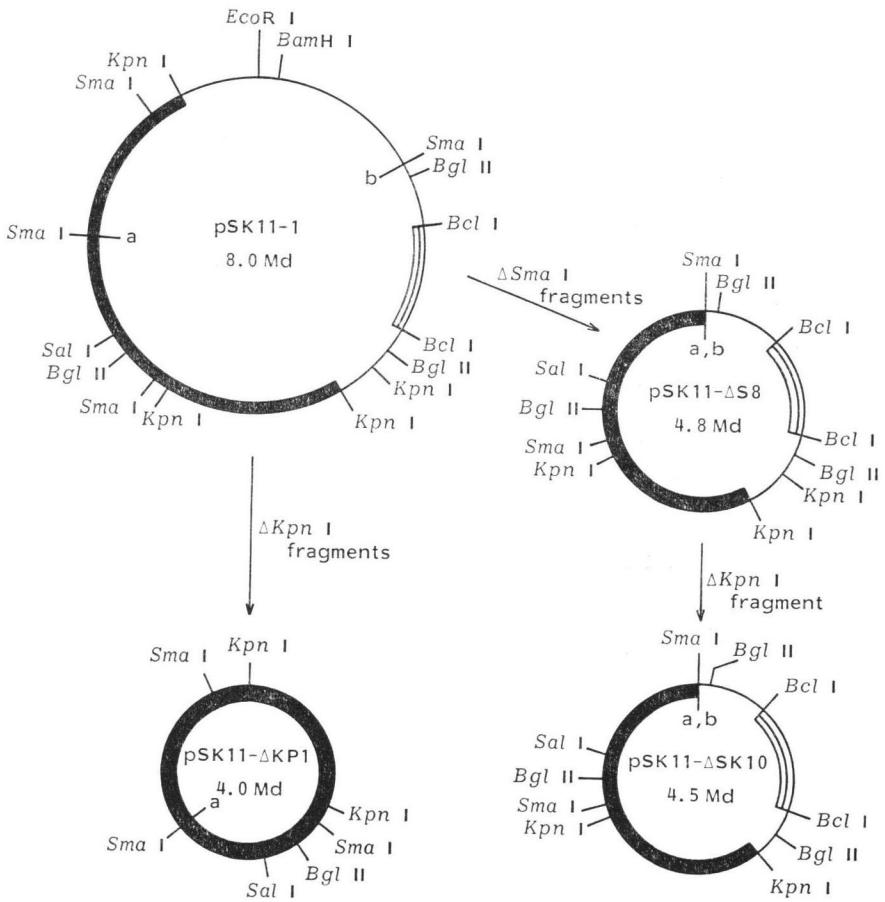


Table 1. Transformation of *S. kasugaensis* and *S. lividans* by pSK11-1 and pSK21-1.

Strain	Plasmid	Transformation frequency (transformants/μg DNA)	Stability (%)
S. kasugaensis G3	pSK11-1	1.8×10^{3}	100
	pSK21-1	3.2×10^{3}	100
	pIJ702	2.0×10^{3}	9.5
S. lividans	pSK11-1	7.3×10^{4}	99.2
	pSK21-1	5.4×10^{4}	100
	pIJ702	8.2×10^{4}	100

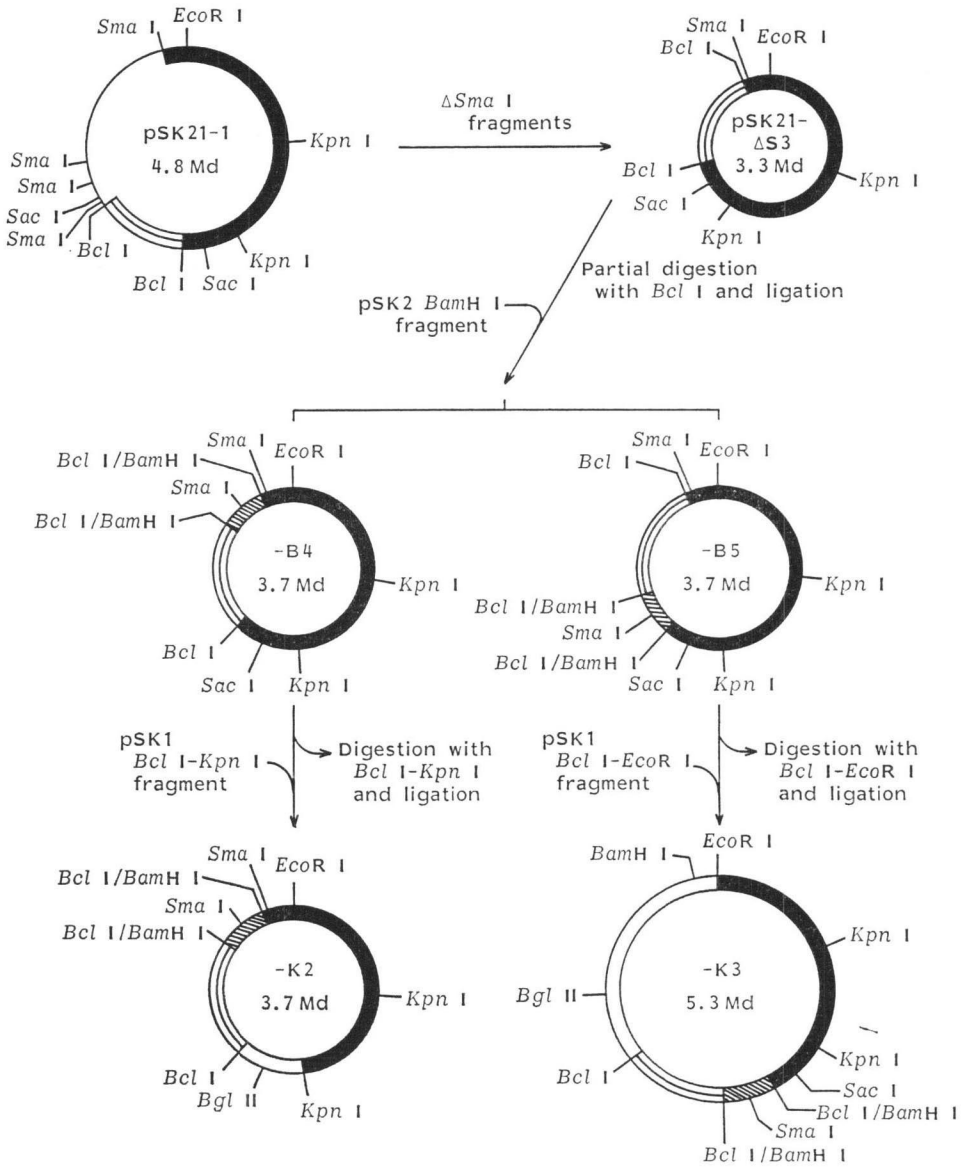
The preparation and transformation of protoplasts, selection of transformants and stability of plasmids are described in the Materials and Methods in the text. Amount of 0.2 μg plasmid DNA was used for the transformation of protoplasts (1×10^{8}).

Fig. 5. Construction of small plasmids from pSK11-1.



thiopeptin-resistant clones obtained by transformation with the ligated mixture of *Sma* I-digested pSK11-1. The pSK11-*⊿*S8 was then converted into a smaller pSK11-*⊿*SK10 by *Kpn* I digestion-ligation, as shown in Fig. 5. On the other hand, when the re-ligated mixture of *Kpn* I-digested pSK11-1 was introduced to G3 protoplasts by transformation, a transformant containing two kinds of small

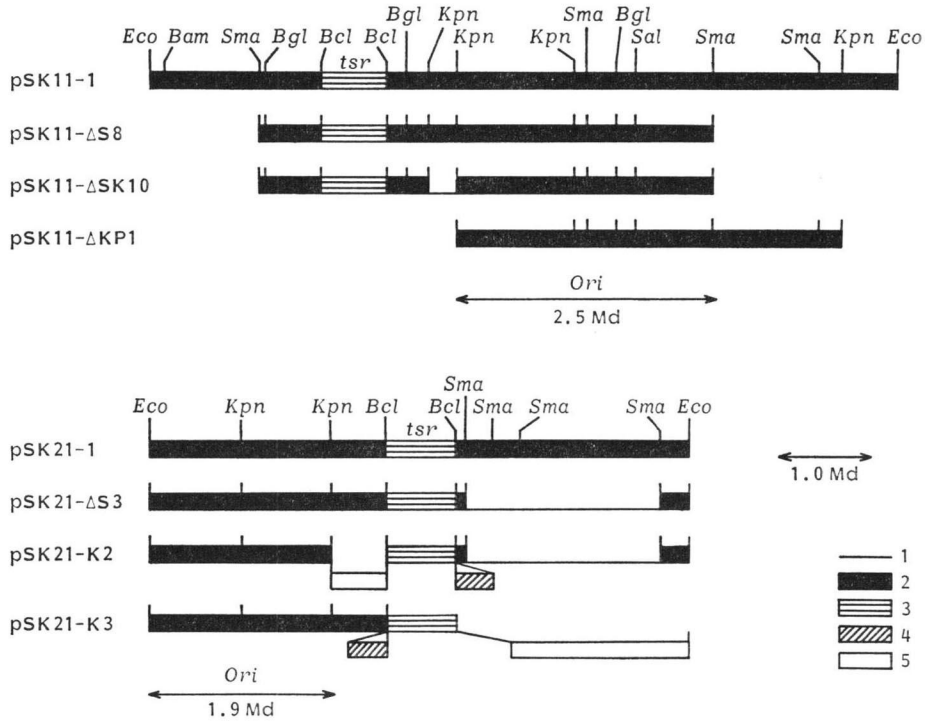
Fig. 6. Construction of small plasmids from pSK21-1 and introduction of multi-insertion sites into them.



plasmids was obtained. The thiopeptin resistance of this clone was very unstable, and pSK11-⊿KP1 was detected from a thiopeptin-sensitive segregant. Analysis of its restriction enzyme sites showed pSK11-⊿KP1 to be lacking two *Kpn* I fragments, including *tsr*. The thiopeptin resistance of the original transformant seemed to be determined by another co-existing plasmid, but this unstable plasmid could not be isolated because of its low yield. Physical mapping of these three small plasmids indicates that the essential region of pSK1 is located between the *Sma* I site and one of the two remaining *Kpn* I site, and that the *Eco*R I and *Bam*H I sites of pSK1 can be used as insertion sites (Figs. 5 and 7).

pSK21-1 was treated with *Sma* I digestion-ligase and introduced into strain G3, and then pSK21-⊿S3 was obtained from the thiopeptin-resistant clones. The structure of pSK21-⊿S3 indicated it was

Fig. 7. Linear expression of restriction enzyme cleavage sites in pSK1 and pSK2 derivatives.
1: Deleted region; 2: region derived from the original plasmids; 3: thiostrepton resistance gene; 4: inserted *Bam*H I fragments derived from pSK2; 5: inserted DNA fragments derived from pSK1.



formed by deletion of three *Sma* I fragments from pSK21-1 (Fig. 6). Since one *Sma* I site remains in pSK21-*⊿*S3, we can use this site for insertion of foreign DNA.

Preparation of pSK21-*⊿*S3 Derivatives Carrying Multi-insertion Sites

pSK21-*⊿*S3 was the smallest plasmid (3.3 Md) among the derivatives from pSK plasmids, and it retained *Eco*R I and *Sac* I sites as single restriction sites in addition to its one *Sma* I site. These sites were determined to be in the dispensable region or not, by means of introduction of certain DNA fragments. In this case, the DNA fragments carrying single sites were used to give more single sites to pSK21-*⊿*S3. The *Bam*H I fragment (0.4 Md) obtained from the dispensable region of pSK2 was inserted into the *Bcl* I sites of pSK21-*⊿*S3 to eliminate one of the two *Bcl* I sites on the plasmid. The physical maps of the composite plasmids obtained were determined and the inserted *Bcl* I fragment was found to exist at one or the other *Bcl* I sites located at both ends of the *tsr* fragment (Fig. 6). Later, this introduced fragment was found to contain one *Sma* I site. Next, a small *Bcl* I-*Kpn* I fragment (0.5 Md) of pSK21-B4 was replaced with the *Bcl* I-*Kpn* I fragment of pSK1 (0.5 Md) to give a single *Bgl* II site, and the obtained pSK21-K2 turned out to have 4 single restriction sites: *Eco*R I, *Sma* I (2 sites, but can use as single site), *Bcl* I and *Bgl* II (Fig. 6).

Similarly, the small *Eco*R I-*Bcl* I fragment (0.3 Md) of pSK21-B5 was replaced with the *Eco*R I-*Bcl* I fragment (1.85 Md) of pSK1, which carried *Bgl* II and *Bam*H I sites. The resultant pSK21-K3 thus possessed 5 single restriction sites: *Eco*R I, *Bam*H I, *Bgl* II, *Bcl* I and *Sac* I (Fig. 6).

Analysis of the physical maps of various plasmids derived from pSK21-1 indicated the *Eco*R I-

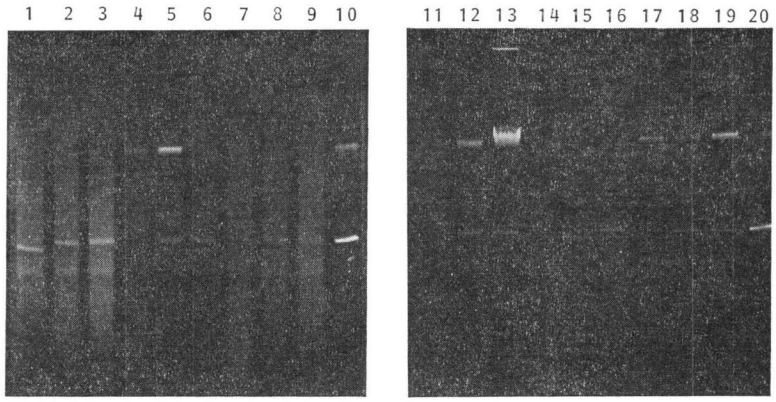
Table 2. Transformation of various species in *Streptomyces* by pSK21-B5.

Strain	Transformation frequency (transformants/μg DNA)	Stability (%)
S. acrimycini IMC S-0206 (ISP 5135)	2.0×10^4	93.7
S. fradiae IFO 3439	1.0×10	92.0
S. griseus ATCC 10137	2.0×10^5	99.6
S. griseus subsp. *solvifaciens* IFO 13689	3.6×10^2	75.2
S. kanamyceticus At-463	2.4×10^3	96.6
S. kanamyceticus At-533	5.0×10	100.0
S. kasugaensis G3	5.2×10^5	100.0
S. kasugaensis M338	1.3×10^3	16.0
S. lividans TK21	2.3×10^4	97.5
S. parvullus ATCC 12434	4.0×10^3	89.9
S. rimosus NRRL 2234	1.0×10^5	100.0
S. vinaceus ATCC 11861	3.5×10^2	100.0
S. venezuelae ISP 5230	2.0×10	100.0

The methods for preparation and transformation of protoplasts were the same as those with *S. kasugaensis* G3 (see Materials and Methods). Amount of 0.3 μg pSK21-B5 was used for the transformation of protoplasts (1×10^8).

Fig. 8. Electrophoresis of plasmids extracted from various species of *Streptomyces*.

1, 2, 3: *S. griseus* subsp. *solvifaciens* IFO 13689. 4, 5, 6: *S. kanamyceticus* AT-463. 7, 8, 9: *S. vinaceus* ATCC 11861. 11, 12, 13: *S. griseus* ATCC 10137. 14, 15, 16: *S. parvullus* ATCC 12434. 17, 18, 19: *S. rimosus* NRRL 2234. 10, 20: pSK21-B5 (control). For each species the first lane is the host itself, and the second and third lanes represent the transformants. The plasmid was detected in all the transformants of *Streptomyces* listed in Table 2.



Kpn I-*Kpn* I fragment (1.9 Md) to be the essential region for the replication and stability of these plasmids (Fig. 7). Therefore, all the single restriction sites in pSK21-B4 and -B5 are indicated to be distributed in the non-essential region of these plasmids.

Host Range and Stability of pSK21-B5 in the Genus *Streptomyces*

To test the host range and stability of one of the constructed plasmids, pSK21-B5 was introduced into various streptomycete protoplasts by application of the transformation procedure for G3 protoplasts. Thiopeptin-resistant clones were detected in 13 of 16 tested species, although the transformation frequency differed with the species (Table 2). In the 3 unsuccessful species, the formation or regeneration of protoplasts had not gone well. The plasmids of these transformants were extracted

and compared with the introduced plasmid by agarose electrophoresis. All transformants except those of *Streptomyces parvullus* ATCC 12434 were proved to carry a plasmid having the same molecular size as pSK21-B5 (Fig. 8). In the case of *S. parvullus*, about 60% of the transformants had a smaller plasmid which seemed to be derived from pSK21-B5 as the result of *in vivo* deletion (Fig. 8 lane 15). *Streptomyces griseus* subsp. *solvifaciens* 13689 (synonymous with *S. albus* G) originally carried a small plasmid, but this plasmid was not detected in the transformants carrying pSK21-B5 (Fig. 8 lanes 1, 2 and 3). The plasmid resident in this strain thus may be incompatible with pSK21-B5. The stability of pSK21-B5 in the transformants of various streptomycetes was tested. The plasmid was found to be maintained stably in all species except for *S. kasugaensis* strain M338, an originally different strain from the *S. kasugaensis* MB273 series. These results indicate that *tsr*-carrying pSK2 derivatives have a wide host range and stability in the genus *Streptomyces*.

Discussion

The pIJ702 established in *S. lividans* was not stably maintained in *S. kasugaensis* G3. This instability is not likely to be particular to *S. kasugaensis*, because pIJ303, one of the pIJ101 series carrying the same replication-origin as pIJ702, is also unstable in *S. albus* G and *S. griseus* ATCC 10137[9]. Time-course study of the proportion of thiopeptin-resistant colonies in liquid culture without thiopeptin reveals that the appearance of sensitive colonies of transformants is dependent on the growth of the strain. This suggests that the pIJ702 is not distributed well to daughter cells of strain G3. The thiopeptin-sensitive mycelia were found to exist in the thiopeptin-containing liquid medium. Mechanism of their existence has not been studied yet, but a ribosomal RNA methylase (capable of protecting ribosomes from binding thiopeptin[7]) or the methylated ribosomes might remain for a while in the cells which had lost the pIJ702.

pSK11-1 and pSK21-1, constructed from the *tsr* fragment and pSK1 or pSK2, were stably maintained in *S. kasugaensis* G3, as expected. The protoplasts of *S. kasugaensis* and *S. lividans* were transformed at a high frequency by these plasmids. The tyrosinase gene of pIJ702 was not used for insertion into the pSK plasmid because the production of brownish pigment by *S. kasugaensis* was affected by complicated factors. *S. kasugaensis* had been reported to be melanin negative[10], but some progenies regenerated from the protoplasts produced reddish brown pigment into the medium in the presence of tyrosine and certain amino acids.

In order to elucidate the essential regions of pSK1 and pSK2, small plasmids derived from pSK11-1 and pSK21-1 were constructed by *in vitro* deletion achieved by treating with restriction enzymes and ligase. When the re-ligated mixture of *Kpn* I-digested pSK11-1 was introduced into protoplasts of strain G3, a transformant, pSK11-KP1, carrying no *tsr* was detected as coexisting with the unstable plasmid carrying *tsr*. This results indicates that the region governing stable inheritance of the plasmid is present in the pSK11-KP1 molecule. On the other hand, the essential regions, which govern plasmid replication and stability in the host, were determined to be less than 2.5 Md and 1.9 Md in pSK11-1 and pSK21-1, respectively.

The host range and stability of pSK21-B5 were examined using a variety of species in the genus *Streptomyces*. All of the tested protoplasts capable of regenerating were transformed with the plasmid, and the transformants showed high stability, except for *S. kasugaensis* M338. This M338 strain was isolated from a different soil than strain MB273[10]. As for the mechanism of the instability in strain M338, it might be considered that this strain carries a similar plasmid or its integrated form on its chromosomal DNA which is incompatible with replication of the pSK2 series of plasmids. A plasmid has, in fact, been detected in strain M338[20].

An *in vivo* deletion was detected in pSK21-B5 introduced into *S. parvullus* ATCC 12434. Similar *in vivo* deletion was observed with pSK21-B4 and *S. noursei* plasmids when they were introduced into *S. lividans* TK21 (data not shown). KIESER *et al.* reported the *in vivo* deletion of pIJ101 derivatives

introduced into cells of *S. lividans* and *S. rimosus*[6]. It seems likely that there are some streptomycetes which frequently cause deletion of the introduced plasmids. The plasmids (pSK21-⊿S3, B4 and B5) constructed in the these experiments have the thiostrepton resistance gene as a selective marker, multi-insertion sites, a wide host range and high stability in various species of *Streptomyces* in addition to being small in size and having a high copy number. Therefore, they should prove to be useful as cloning vectors in the genus *Streptomyces*. Recently, AKAGAWA found out pock-forming derivatives from pSK1 and pSK2[21], but the composite plasmids (pSK21-⊿S3, B4 and B5) did not show any pock-forming ability.

Acknowledgments

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