MUTUAL RELATION OF THREE POCK-FORMING PLASMIDS RESIDENT IN *STREPTOMYCES NOURSEI*

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(Received for publication March 29, 1984)

From *Streptomyces noursei* B3, two plasmids — designated pSCY5 and pSCY6 — were isolated in addition to the known plasmid pSCY3. Strains carrying one or more of these plasmids generated pocks on a lawn of the plasmid-free strain, *S. noursei* KL3. The pocks elicited by pSCY3 and pSCY5 belonged to the A type; *i.e.*, showed a clear inhibition zone, while those produced by pSCY6 were of the B type; *i.e.*, exhibited a turbid inhibition zone. The strains carrying either pSCY3 or pSCY5, were also capable of forming pocks on a lawn of *S. noursei* 6T-11 harboring pSCY6, and *vice versa*. However, pock formation was not observed between strains harboring pSCY3 and strains carrying pSCY5. The endonuclease cleavage maps of these plasmids revealed that pSCY3 differed clearly from that of pSCY6, whereas pSCY5 was found to be a hybrid plasmid consisting of the entire pSCY3 plasmid and an 8.4 Md or longer fragment originating from pSCY6. The pocks elicited by pSCY5 were much smaller than those produced by pSCY3. Transformation experiments showed that pSCY6 elicited pocks in *Streptomyces lividans* as well as in *S. noursei*, whereas the pSCY6 transformants of *S. lividans* failed to produce pocks on a lawn of plasmid-free *S. noursei*.

Numerous plasmids have recently been isolated from streptomycetes. Their functions have been examined from the point of view of antibiotic biosynthesis, regulation of antibiotic production, resistance to antibiotics by the producing organisms, relation with pleiotropic phenotypes, *etc.*[1~5]. However, their role is still poorly understood, except in the case of the plasmid carrying the genetic determinants for methylenomycin biosynthesis and resistance, sex plasmids and self-transmissible plasmids capable of producing pocks[5~9]. Pock formation, initially designated as lethal zygosis, was described by Bibb *et al.*[7] in the case of SCP2 and SCP2* plasmids of *Streptomyces coelicolor* A3 (2)[8] and this phenomenon is thought to be characteristic of several streptomycete plasmids[10~12]. Subsequently, Kieser *et al.*[13] assigned the probable pock-determinant region to a part of pIJ101 which was named the "spread" region. In addition to the detailed biological studies of plasmids, Bibb *et al.*[14] showed that SLP1 plasmids in *Streptomyces lividans* were formed by excision of DNA segments from the chromosome of *S. coelicolor* during mating. On the other hand, Sermonti *et al.*[15] predicted that *S. coelicolor* might contain a "jumping" gene capable of transposing onto plasmid SCP1. In spite of these findings, there remains much to be studied about the biological and physical properties of streptomycete plasmids.

S. noursei 2217-G1 is a producer of cycloheximide and nystatin, and carries several types of plasmids. Hayakawa *et al.*[16] reported the isolation of plasmids pSCY1 and pSCY2 from this streptomycete, but their functions were not elucidated. Takeda *et al.*[17] of our laboratory, described pSCY2, pSCY3 and pSCY4 in *S. noursei* variant strain B3 and constructed the endonuclease cleavage map of pSCY3 which had been transformed into *Escherichia coli* using pACYC184 as the vector. We have been interested in studying the functions and natural variations of the plasmids in *S. noursei* with respect to pock and spore formation, resistance to various agents and antibiotic biosynthesis. In this paper we describe the pock-forming properties of pSCY3 and two newly isolated plasmids, pSCY5 and pSCY6. We also

Table 1. *Streptomyces* strains employed.

Strain		Plasmids	Remarks
Streptomyces noursei	B3	pSCY3, pSCY5, pSCY6	TAKEDA *et al.*
〃	P1	pSCY6	from regenerants of B3 protoplasts
〃	P2	pSCY3=pSCY6<pSCY5	from regenerants of B3 protoplasts
〃	A38	pSCY6	from AC-resistants of B3
〃	N24, N49	—	from NB-resistants of B3
〃	P2S1	pSCY5	from small pocks of P2
〃	KL3	—	from regenerants of P1 protoplasts
〃	3T-11	pSCY3	from transformants of KL3 by pSCY3
〃	5T-11	pSCY5	from transformants of KL3 by pSCY5
〃	6T-11	pSCY6	from transformants of KL3 by pSCY6
S. lividans			
〃	1326	—	HOPWOOD *et al.*
〃	6TL-11	pSCY6	from transformants of 1326 by pSCY6

pSCY4 could not be detected in the present study. SLP2 and SLP3[9] were not referred to in the present study.
= and < mean amounts of plasmids.
AC: Acridine orange. NB: Novobiocin.

present their restriction maps. A pathway for intracellular plasmid generation of pSCY5 is proposed.

Materials and Methods

Media

ASH medium consisting of maltose 1.0%, yeast extract 0.3%, wood ash 0.03% and agar 1.0%; pH 7.2 was used for slant culture and pock formation. GPY medium consisting of glucose 1.0%, Polypeptone 0.4%, yeast extract 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.1% and glycine 0.1% was used for growth of the organisms under shaking conditions. P3 and R3 media[18] were used for protoplast regeneration of the streptomycetes used.

Bacterial Strains

S. noursei B3 isolated by TAKEDA *et al.*[17] from *S. noursei* 2217-G1 was used initially as the wild type strain, and its derivatives, used in the present study, are listed in Table 1. *S. lividans* strain 3131 harboring pIJ702[19] (provided by Prof. E. KATZ, Georgetown University, Schools of Medicine and Dentistry) and the pIJ702-free strain obtained from the strain 3131 harboring the plasmid by protoplast regeneration were employed in the experiments.

DNA Preparation

Chromosomal DNA of *S. noursei* was prepared from strain KL-3 (plasmid-free) using the method of OKANISHI *et al.*[20]. For small-scale preparation of plasmid DNA from *S. noursei*, the alkaline lysis procedure of CHATER *et al.*[21] was modified as follows: protoplasts prepared from a culture grown in 5 ml of GPY medium were suspended in 0.5 ml of P3 medium. The suspension was combined with 0.5 ml of hot lytic solution[21] and mixed well for 1~2 minutes at room temperature. The lysate was mixed with 0.3 ml of 2.0 M Tris-HCl buffer, pH 7.0, and then with 1.0 ml of phenol - chloroform solution[21]. After vortexing for 30 seconds, the upper phase was obtained by centrifugation and an aliquot was applied to a horizontal agarose gel slab. This modified procedure was also found to be applicable to *S. lividans* strains containing plasmids. Large-scale isolation of plasmids was carried out by the method of OKANISHI *et al.*[22]. Separation of individual plasmids from their mixture or isolation of individual digested DNA fragments was performed using preparative agarose gel electrophoresis. DNA was electrophoretically eluted from regular agarose gel pieces and extracted by phenol - chloroform[23] treatment according to the method of MANIATIS *et al.*[23].

Elimination of Plasmids from *S. noursei*

Three methods were applied for eliminating plasmids from *S. noursei* B3. (1) Acridine orange

treatment: Strain B3 was cultured with shaking in the dark at 28°C for 3 days in 25 ml of GPY medium containing 40 μg acridine orange/ml. The culture (0.1 ~ 0.2 ml) was spread on GPY agar medium (agar 1.8%) after appropriate dilution. (2) Novobiocin treatment: Mycelium from a shaken culture (0.1 ~ 0.2 ml) was plated on GPY agar medium containing 5 ~ 10 μg novobiocin/ml and incubated at 28°C. Novobiocin was a gift from The Upjohn Company (Japan). (3) Protoplast regeneration: The protoplasts were regenerated on R3 medium by the method of SHIRAHAMA *et al.*[18]. The colonies were collected and spread on ASH medium. With each method, candidates for cured strains were screened from the colonies with abundant spores and submitted to the alkaline lysis procedure and agarose gel electrophoresis to select plasmid-free strains.

Conditions for Pock Formation

Putative donor and recipient strains were separately incubated with shaking in GPY medium at 28°C for 40 ~ 48 hours. The donor culture was appropriately diluted with MS solution ($MgSO_4 \cdot 7H_2O$ 0.05% and NaCl 0.5%), and 0.1 ~ 0.2 ml of a suitable dilution was spread with an equal volume of the undiluted recipient culture on a plate of ASH medium. Observations were made for pocks on the plate after incubation for 3 days at 28°C.

Digestion of DNA with Restriction Endonucleases and Agarose Gel Electrophoresis

In accordance with the method of TAKEDA *et al.*[17], DNA samples were digested for 1 ~ 2 hours at 37°C (50°C for *Bcl* I) with restriction endonucleases. Incubations were terminated by heating at 70°C for 10 minutes. Electrophoresis was performed at 100 V in a horizontal gel slab of 0.8% agarose in Tris-phosphate buffer (89 mM Trizma base, 2.5 mM EDTA-Na_2 and 23 mM H_3PO_4), pH 8.3. The molecular weights of DNA fragments were measured with reference to the *Hin*d III digest of bacteriophage lambda DNA.

Hybridization

Hybridization was carried out with the Southern blotting technique as modified by WAHL *et al.*[24]. The largest *Bam*H I fragment (11.8 Md) of pSCY5 to be used as probe was electroeluted from the agarose slab gel, as described above; a radiolabeled fragment was prepared with ^{32}P using a Nick Translation Kit (Amersham). After hybridization, filters were exposed to Kodak X-O films at −20°C for 60 ~ 80 hours for autoradiography.

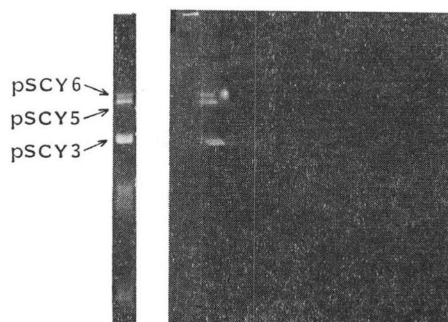
Transformation

Protoplasts of *S. noursei* KL3 and the pIJ702-free strain of *S. lividans* 3131, as recipients of plasmid DNA, were prepared by the procedure of SHIRAHAMA *et al.*[18] and transformed with plasmid DNA according to the method of CHATER *et al.*[21]. Transformants harboring plasmids were detected by their pock-forming abilities as follows: Protoplasts were grown at 28°C for 7 ~ 10 days embeded in the upper layer of a double-layered regeneration medium (R3)[18]. The upper layer containing regenerants was separated from the lower layer with a glass rod, placed into GPY liquid medium and then cultivated under shaking conditions with a spring coil for 10 ~ 20 hours prior to the pock assay. The diluted culture was spread on a plate of ASH medium together with the corresponding recipient strain and then pock-forming transformants were isolated from the colonies growing in the centers of the pocks. In the case of thiostrepton-resistant transformants, the protoplasts submitted to the transformation procedure were incubated for 15 ~ 20 hours and then the surface of the medium was overlaid with 5 ml of molten (30°C) R3 medium containing 250 μg of thiopeptin and 0.5% of low-melting point agar. The resistant clones were detected after incubation for 5 ~ 7 days at 28°C. Thiopeptin, used instead of thiostrepton, was a kind gift from Fujisawa Pharmaceutical Co., Ltd., Japan.

Hybrid Plasmids

DNA of pIJ702 was digested with *Bcl* I and submitted to agarose gel electrophoresis for isolation of the *Bcl* I fragment containing the thiostrepton resistance determinant. The *Bcl* I fragment of 1.6 Md was electrophoretically eluted from the agarose gel and extracted by phenol - chloroform[23] treatment as described above. A partial *Bam*H I digest or a *Bcl* I digest of pSCY3 was ligated at 4°C for 48 hours with the thiostrepton-resistant fragment. The ligated mixture was introduced into the protoplasts of the

Fig. 1. Two-dimensional agarose gel electrophoresis[13] of plasmid DNA's isolated from *S. noursei* B3.



pIJ702-free strain of *S. lividans* by the transformation procedure and resistant transformants were selected as described above.

Results

Plasmids in *S. noursei* B3

Using the modified alkaline extraction method, agarose gel electrophoresis revealed that there were three plasmid-like bands in the DNA preparation derived from *S. noursei* B3. This observation was confirmed by two-dimensional agarose gel electrophoresis[21] (Fig. 1). One of them was identified as pSCY3, whereas the other two were previously unknown and named pSCY5 and pSCY6. Additional DNA bands which moved faster than the three plasmids in the first dimension were not considered to be plasmids, based on their higher mobility in the second dimension.

Elimination of Plasmids from *S. noursei* B3

Preparation of a plasmid-free strain is required not only to obtain a recipient host for transformation experiments with plasmid DNA but also to carry out DNA cloning experiments. Three curing procedures were employed to eliminate the plasmids from *S. noursei* B3. The numbers of colonies screened by the three procedures were 49 by acridine orange treatment, 50 using novobiocin, and 30 by protoplast regeneration. According to a detailed analysis, two novobiocin-resistant strains (N24 and N49) were found to be plasmid-free. Only pSCY6 was present in one acridine orange-resistant strain (A38) and in one regenerant (P1) obtained from the protoplasts. Some regenerants showed an altered plasmid composition in comparison with strain B3. For example, strain P2 contained larger amounts of pSCY5 but smaller levels of pSCY3 and pSCY6. Repeated protoplast regeneration of strain P1 yielded 12 plasmid-free isolates; strain KL3 was chosen from these isolates because of its ability to sporulate abundantly These results are summarized in Table 2.

Detection of Pock Formation with the Plasmid-free Strain KL3 (*S. noursei*)

The ability to generate a pock phenotype is one of the properties associated with many *Streptomyces* plasmids. This was examined using strains of *S. noursei* harboring plasmid(s) as donors and plasmid-free strains as recipients. All the plasmid-bearing strains elicited the pock phenotype when incubated on a lawn of the plasmid-free strain KL3, but not on strains N24 and N49. The reason for a lack of pock formation in the case of the latter two strains has not yet been established. Depending on the clarity of

Table 2. Plasmid elimination in *S. noursei* B3.

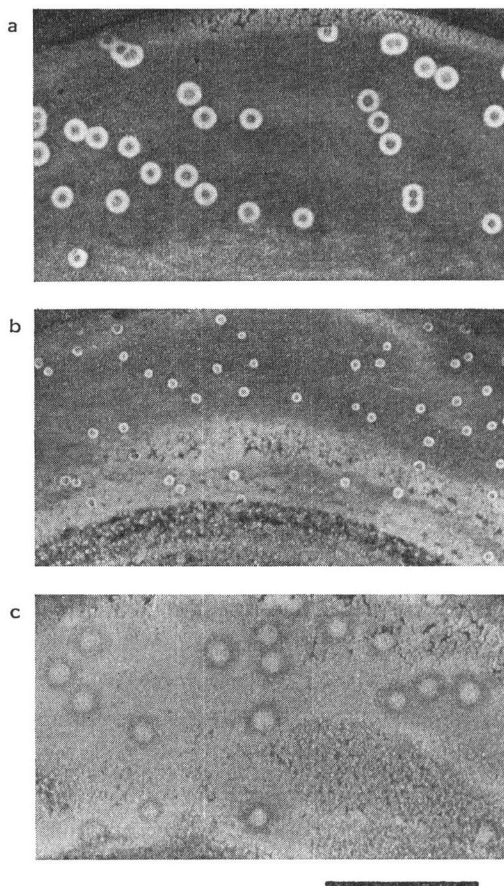
Test	Curing treatment	Number of colonies			
		Grown	Sporulated	Cured	Partially cured
1	Acridine orange (40 μg/ml)	49	43	0	1 (A38, pSCY6$^+$)
	Novobiocin (5~10 μg/ml)	50	22	2 (N24, N49)	0
	Protoplast regeneration	30	14	0	1 (P1, pSCY6$^+$)
2	Protoplast regeneration of strain P1		40	12 (KL1 to KL12)	

the pocks, pock morphology was classified into three types (A, B and As). A-type pocks (clear inhibition zones) were always observed when the donor strain was B3 which carried pSCY3, pSCY5 and pSCY6. B-type pocks (turbid or opaque) were caused by strains P1 and A38 which harbored pSCY6. On the other hand, As-type pocks (much smaller than A-type pocks) were initially recognized among normal A-type pocks when the donor was strain P2 which carried predominantly pSCY5 and lesser amounts of pSCY3 and pSCY6. The three pock types are illustrated in Fig. 2.

Fig. 2. Pocks produced by strain B3 and derivatives on a lawn of plasmid-free strain KL3.

a: A-type pocks (strain B3); b: As-type pocks (strain P2S1); c: B-type pocks (strain P1).

The bar represents 10 mm.



Following experiments with strain KL3 as host, many strains were isolated from the perimeters of the pock zones using fine wooden tips and then their plasmid composition and pock-forming type were examined. Strains isolated from A-type pocks (derived from strain B3) usually harbored pSCY3, pSCY5 and pSCY6, but some strains lacked pSCY5 (strain K4211 is representative). All these isolates also elicited the A-type pock phenotype on strain KL3. Strains derived from B-type pocks carried only pSCY6 and formed B-type pocks on the recipient strain KL3. Strains obtained from As-type pocks had pSCY5 alone and produced As-type pocks (strain P2Sl is representative). These findings suggested that the specific pock phenotype depended on the particular plasmid: Thus, strains bearing pSCY5 alone formed As-type pocks, whereas strains carrying only pSCY6 produced B-type pocks. By contrast, all of the strains carrying pSCY3 elicited A-type pocks whether pSCY5 or pSCY6 was present or not.

Mutual Relation of the Three Plasmids on the Expression of Pocks

Strain KL3 was transformed with each of the three plasmids (pSCY3, pSCY5 or pSCY6) to establish which plasmid(s) was (were) responsible for a particular pock phenotype. The three plasmids were isolated from agarose gels by electroelution and extracted by phenol - chloroform treatment as described above. After introduction into protoplasts of strain KL3, the regenerants were subjected to pock assay and transformants were isolated from the growths in the center of pocks. The plasmids extracted from those strains were identified by gel electrophoresis after digestion with restriction enzymes. Representative strains which carried pSCY3 (3T-11), pSCY5 (5T-11) and pSCY6 (6T-11), respectively, were analyzed in more detail.

As Table 3 shows, strains 3T-11 (pSCY3), 5T-11 (pSCY5) and 6T-11 (pSCY6) produced type A, As

Table 3. Pock formation between various strains of *S. noursei*.

Donor strain of *S. noursei*	Plasmid pSCY	Recipient strain of *S. noursei*				
		KL3	B3 pSCY3, 5, 6	3T-11 pSCY3	P1 & 6T-11 pSCY6	P2S1 & 5T-11 pSCY5
B3	3, 5, 6	+(A)	−	+	+(A)	+
3T-11	3	+(A)	−	−	+(A)	−
P1 & 6T-11	6	+(B)	−	+	−	+
P2S1 & 5T-11	5	+(As)	−	−	+(As)	−

+; Pocks, −; no pocks.
A: A-type pocks, B: B-type pocks, As: As-type pocks.

and B pock phenotypes on strain KL3, respectively. Furthermore, strains B3 (pSCY3, pSCY5 and pSCY6), P1 (pSCY6) and 6T-11 (pSCY6) which carried commonly pSCY6 caused pocks on a lawn of strain 3T-11 (pSCY3) or strain 5T-11 (pSCY5), but we could not determine their pock types because of poor sporulation of these lawn strains. Strains harboring pSCY5 (5T-11 and P2Sl) did not form pocks on a lawn of a pSCY3-bearing strain (3T-11), and *vice versa.* On the other hand, strains carrying either pSCY3 or pSCY5 could express pock formation on recipient strains harboring pSCY6 alone, and *vice versa.*

Physical Maps and Molecular Relationship among the Three Plasmids

The restriction endonuclease cleavage maps of pSCY5 and pSCY6 were established and the results are presented together with the map for pSCY3 (Fig. 3)[17]. On the basis of the three restriction maps, pSCY6 differs clearly from pSCY3, while pSCY5 appears to consist of the entire DNA molecule of pSCY-3 with at least an 8.4 Md fragment inserted between the *Sac* I and *Bam*H I sites (between sites 13 and 14) in pSCY3. The 8.4 Md fragment, which also is present in pSCY6, includes the region containing the *Pvu* II (site a′) and the *Sac* I recognition site (site m′).

Hybridization studies were carried out to establish the homology of the 8.4 Md fragment in both pSCY5 and pSCY6. The largest *Bam*H I-A fragment (11.8 Md; sites 32–a–m–34) of pSCY5 was used as a probe, due to the fact that it included the whole 8.4 Md segment and could be readily isolated. The probe hybridized with fragments of pSCY6 digested with *Sac* I, *Bgl* II or *Pvu* II, but did not show homology with the *Bam*H I digest of chromosomal DNA from strain KL3. The region in pSCY6 which supposedly incorporated the 8.4 Md fragment was found to include all the strongly-hybridized fragments; That is, *Bgl* II-A, C and F (sites j′–54–44, sites c′–j′ and sites 52–c′), *Pvu* II-C, D, E and F (sites e′–k′, sites k′–l′, sites a′–e′), and *Sac* I-C, D, E, F and F′ (sites i′–m′, sites d′–g′, sites h′–i′, sites g′–h′ and sites b′–d′) in Figs. 3 and 4. Evidence for the presence of the 8.4 Md fragment in both pSCY5 and pSCY6 was provided by the hybridization studies and the endonuclease cleavage maps. Furthermore, it appeared that this common region was not derived from the chromosomal DNA of *S. noursei.* On the other hand, the probe also hybridized strongly with the *Bam*H I-C fragment (sites 29–32) of pSCY5, as shown in lane 3′ in Fig. 4, suggesting that a homologous region which was not found in their restriction maps was present in parts of both the probe (*Bam*H I-A) and the *Bam*H I-C fragment.

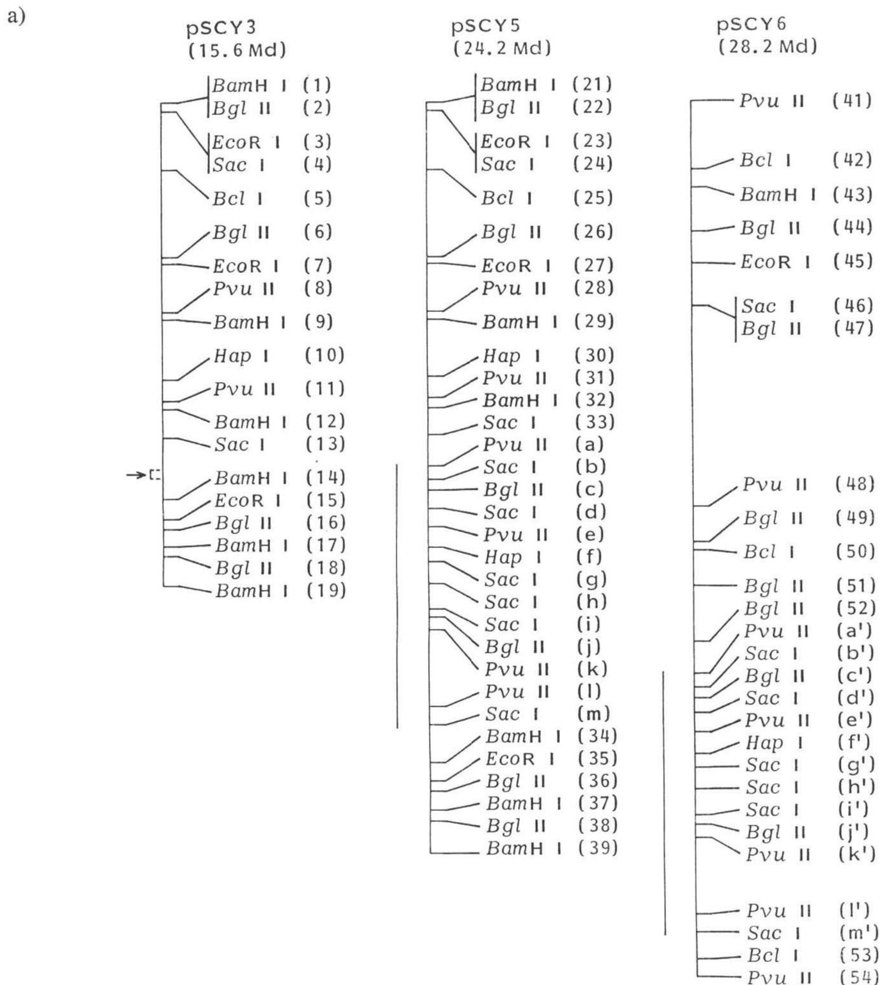
Transformation of *S. lividans* by Plasmids

Transformation of plasmid-free *S. lividans* protoplasts was carried out with the individual plasmids, pSCY3, pSCY5 and pSCY6, respectively, and pock-forming transformants were examined subsequently on a lawn of *S. lividans.* Of the three plasmids, only pSCY6 DNA elicited pock formation (strain 6TL-

Fig. 3. Restriction endonuclease cleavage maps of pSCY3, pSCY5 and pSCY6 (a) and fragment sizes of the plasmids (b).

These closed circular plasmid DNAs are figured as linear form. The lines along pSCY5 and pSCY6 indicate the about 8.4 Md fragment. The solid arrow along with pSCY3 shows the probable region where the 8.4 Md fragment is inserted in generating pSCY5. The exact inserted position of the fragment in pSCY3 could not be determined.

a)



b) Distance to each restriction enzyme site.

(pSCY3) Md	(pSCY5)* Md	(pSCY6)** Md	(8.4) Md	(pSCY3) Md	(pSCY5)* Md	(pSCY6)** Md	(8.4) Md
1. 0.0	21. 0.0	41. 0.0	a, a′. 0.0	11. 9.6	31. 9.6	51. 15.6	k, k′. 5.3
2. 0.0	22. 0.0	42. 2.2	b, b′. 0.4	12. 9.9	32. 9.9	52. 17.4	l, l′. 7.8
3. 0.3	23. 0.3	43. 2.8	c, c′. 0.7	13. 10.8	33. 10.8	53. 27.6	m,m′. 8.4
4. 0.3	24. 0.3	44. 4.2	d, d′. 1.3	14. 12.8	34. 21.4	54. 28.2	
5. 2.2	25. 2.2	45. 5.2	e, e′. 1.9	15. 13.4	35. 22.0		
6. 5.0	26. 5.0	46. 6.6	f, f′. 2.6	16. 13.7	36. 22.3		
7. 5.2	27. 5.2	47. 6.6	g, g′. 3.0	17. 14.3	37. 22.9		
8. 6.8	28. 6.8	48. 13.0	h, h′. 3.7	18. 14.6	38. 23.2		
9. 7.0	29. 7.0	49. 14.2	i, i′. 4.6	19. 15.6	39. 24.2		
10. 8.9	30. 8.9	50. 14.4	j, j′. 4.9				

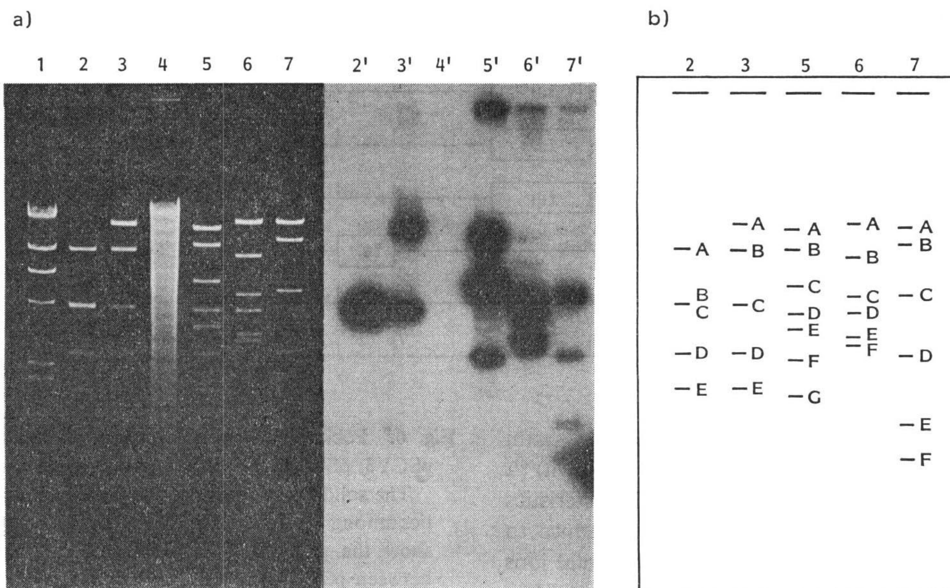
* 33–a, m-34; not determined.

** 52–a′, 1.0 Md; m′-53, 0.8 Md.

Fig. 4. Digestion patterns of the three plasmids and the chromosomal DNA by restriction endonucleases (lanes 1～7) and the corresponding autoradiograms after hybridization with ^{32}P-labeled *Bam*H I-A fragment (sites 32–34 in Fig. 3) of pSCY5 (lanes 2′～7′).

a) Lane 1: lambda DNA digested with *Hin*d III; lanes 2 & 2′: pSCY3 digested with *Bam*H I; lanes 3 & 3′: pSCY5 digested with *Bam*H I; lanes 4 & 4′: chromosomal DNA digested with *Bam*H I; lanes 5 & 5′: pSCY6 digested with *Bgl* II; lanes 6 & 6′: pSCY6 digested with *Pvu* II; lanes 7 & 7′: pSCY6 digested with *Sac* I.

b) Schematic view of lanes 2, 3, 5, 6 and 7 from a). Each digest is alphabetically named in order of decreasing size. Lane 2: digest of pSCY3 with *Bam*H I; lane 3: digest of pSCY5 with *Bam*H I; lane 5: digest of pSCY6 with *Bgl* II; lane 6: digest of pSCY6 with *Pvu* II; lane 7: digest of pSCY6 with *Sac* I.



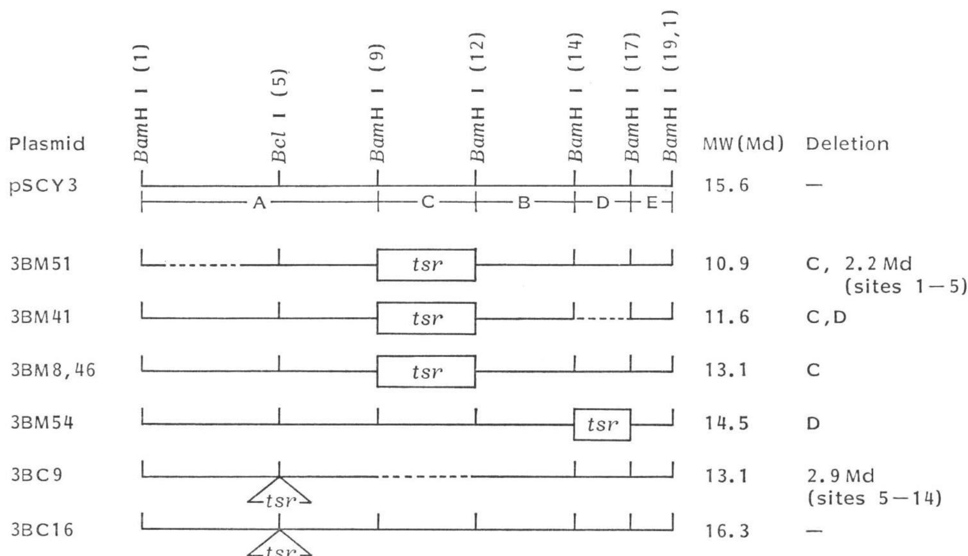
11 is representative). To establish whether transformation of *S. lividans* could be accomplished with pSCY3, a partial *Bam*H I or a *Bcl* I digest of pSCY3 were ligated with the *Bcl* I fragment encoding the thiostrepton-resistance (*tsr*) determinant derived from pIJ702. After transformation of *S. lividans* protoplasts with ligation mixtures, thiostrepton resistant colonies were selected on regeneration medium and the resulting hybrid DNAs were isolated and characterized by restriction endonuclease treatment and gel electrophoresis, as shown in Fig. 5. The *tsr* determinant replaced the *Bam*H I-C fragment (sites 9–12 in Fig. 3) and the *Bam*H I-D (sites 14–17) in pSCY3, giving rise to plasmids 3BM8, 41, 46 and 51, and 3BM54, respectively. Insertion of the *tsr* determinant into the *Bcl* I site (site 5) gave plasmids 3BC9 and 16. Some deletions were observed in plasmids (3BM41, 3BM51 and 3BC9) as seen in Fig. 5. Reintroduction of these plasmids into *S. lividans* gave rise to *tsr* colonies with high frequency, but strains carrying these recombinant plasmids did not form pocks.

Discussion

As reported by OKANISHI[25] and HOPWOOD *et al.*[9], protoplast regeneration proved to be an effective technique for eliminating plasmids from *S. noursei* B3. The procedure also resulted in a marked change in the plasmid composition of a strain (*e.g.*, strains P1, P2 and KL3) in comparison with the parent, *S. noursei* B3. These results are similar to those described by FURUMAI *et al.*[5] in studies with *Streptomyces kasugaensis*. It is also of interest to point out that the plasmid-free strain, KL3, produced almost the

Fig. 5. Hybrid plasmids between pSCY3 and the *Bcl* I fragment encoding the thiostrepton resistance gene (*tsr*) from pIJ702.

The dotted lines indicate the deleted region. Restriction enzyme sites and their numbers refer to those of pSCY3 in Fig. 3. Fragments A, B, C, D and E represent the regions between the corresponding sites and their fragment sizes are 7.0, 2.9, 2.9, 1.5 and 0.7 Md, respectively.

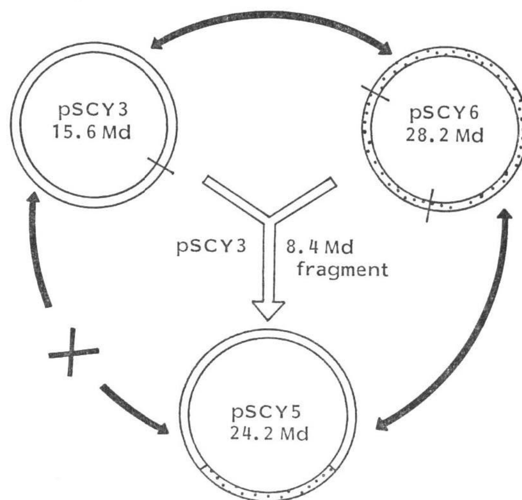


same amounts of both cycloheximide and nystatin as strain B3 on agar pieces (data not shown)[26]. Moreover, strain KL3 had the same characteristics as the parent strain in terms of resistance to various antibiotics as well as to heavy metal ions such as cupric, ferrous or mercuric ions (data not shown). Thus, it seems likely that the three plasmids, pSCY3, pSCY5 and pSCY6, are not involved in the metabolic functions of *S. noursei.*

Plasmids pSCY3, pSCY5 and pSCY6, respectively, were shown to possess genetic information for the A, As and B type pock phenotypes (*S. noursei*). A-type pocks on strain KL3 were produced by strains carrying not only pSCY3 but also both pSCY3 and pSCY6, irrespective of the presence of pSCY5. Many strains were isolated from the perimeters of the A-type pocks produced by strain B3. Almost all of them carried pSCY5 and pSCY6 in addition to pSCY3, suggesting that the three plasmids were transferred together into the recipient cells. Therefore, the A-type pock phenotype exhibited by strain B3 when all the three plasmids are simultaneously present may result from the repressed expression of the other pock phenotypes (As and B). However, the reason for the predominance of the A-type over the other pock phenotypes has not yet been established. Moreover, pSCY3- or pSCY5-carrying strains could form pocks on pSCY6-bearing strains, and *vice versa.* KIESER *et al.*[13] demonstrated that expression of pocks induced by pIJ101 was regulated by genetic determinants for "spread" ability and inser-

Fig. 6. Pock-forming and molecular relations among pSCY3, pSCY5 and pSCY6 in *S. noursei.*

The solid arrows indicate the pock-forming abilities among the three plasmids and the hollow arrows show the probable formation process of pSCY5 between pSCY3 and the 8.4 Md fragment derived from pSCY6.



tion of a foreign DNA segment into the determinant region resulted in smaller pocks. Plasmid pSCY5 was shown to be a composite plasmid consisting of pSCY3 and an 8.4 Md fragment from pSCY6 and elicited much smaller A-type pocks (As) compared with those produced by pSCY3. It seems likely that the insertion of the 8.4 Md fragment into pSCY3 gave rise to smaller A-type pocks. Plasmid pSCY-6 appears to be different from both pSCY3 and pSCY5 in their endonuclease cleavage maps and pock-forming properties as demonstrated by the conjugation experiment. In addition, pSCY6, in contrast to pSCY3 and pSCY5, could elicit pocks in *S. lividans*.

A putative plasmid which is devoid of the 8.4 Md fragment from pSCY6 has not yet been detected and this fragment was not located in the chromosomal DNA of *S. noursei*. Therefore, plasmid pSCY5 is considered to have been constructed *in vivo* by insertion into pSCY3 of the 8.4 Md fragment derived from pSCY6 (Fig. 6). It is also not evident whether or not such an *in vivo* recombination between plasmids is prevalent among streptomycetes. However, this type of recombination gives one possible means for generating new plasmids within a cell. Recently, similar *in vivo* recombination between plasmids was suggested by NAKANO *et al.*[27] and SHIGYO *et al.*[28] but they were not shown that recombination actually had occurred.

In this paper, we have clarified the relationship of the three plasmids resident in *S. noursei* based on pock-forming properties, restriction endonuclease cleavage maps and DNA hybridization experiments. Plasmids pSCY3 and pSCY6 are compatible with each other and look like high copy-number plasmids based on their high extraction yields compared with pIJ702 in *S. lividans*. Both plasmids can also replicate in *S. lividans*.

Acknowledgments

The authors express deep thanks to Drs. K. TAKEDA, T. FURUMAI and H. AKAGAWA for their advice and discussions during the present work. This work was supported, in part, by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency, Japan, and by a Lilly Research Grant from the Japan Antibiotics Research Association.

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