

PLASMID VARIABILITY IN THE ISTAMYCIN PRODUCING STRAINS  
OF *STREPTOMYCES TENJIMARIENSIS*

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Three strains of istamycin-producing *Streptomyces tenjimariensis* were isolated over a period of time from soils at the same location and were found to have three different types of plasmid profiles. Protoplast fusion between two of these strains provided a clone harboring a smaller plasmid not present in the parent strains. None of the plasmids had restriction sites for *EcoR* I and *Hind* III. Most of the plasmids had one or two restriction sites for *Bam*H I, *Bcl* I, *Bgl* II, *Kpn* I, *Pst* I and *Pvu* II, and more than two restriction sites for *Sal* I and *Sst* II. Plasmid restriction maps and SOUTHERN hybridization experiments revealed that pST2, pST12 and pST22 were identical, as were pST10 and pST20. In addition, it was revealed that pST1\*, pST1, pST11 and pST21 were related to each other.

Plasmids in streptomycetes have primarily been investigated in terms of potential involvement in antibiotic production<sup>1)</sup> and as host vector systems.<sup>2,3)</sup> Besides these orientations, there are reports dealing with the structural relationship between specific plasmids and between other plasmids and the chromosome.<sup>4-6)</sup> However, there has been only one report dealing with the relationship among plasmids of naturally occurring strains within a certain *Streptomyces* species.<sup>7)</sup>

We isolated several strains of *S. tenjimariensis*<sup>8)</sup> which produce aminoglycoside antibiotics, istamycins, and harbor several plasmids. These strains were isolated from soils at the same location in a marine environment during each of three successive years. Three types of strains having different plasmid profiles, though the bands showing the same migration were also detected among their profiles, were found. We characterized the plasmids from these strains to determine their structural relationship. The relationship between the plasmids might provide some insight into the genetic changes that take place in streptomycetes in nature.

### Materials and Methods

#### Strains

*Streptomyces tenjimariensis* SS-939, SS-980 and SS-1507 were isolated by the authors from soils taken from the shore at Tenjin island (Miura peninsula, Kanagawa Prefecture, Japan) during Aug. 1978 to Feb. 1980 as described previously.<sup>8)</sup> The strains F4, F9 and F12 were obtained by protoplast fusion between strains SS-939 and SS-1507.

#### Culture Condition and Media

Strains of *S. tenjimariensis* were grown for 48~72 hours at 27°C in half strength ZoBELL medium<sup>9)</sup>; glucose 0.5%, Polypeptone 0.25%, yeast extract (Difco) 0.05% and Artificial Sea Water (Jamarin Lab., Osaka, Japan) 50%.

#### Isolation of Plasmid DNA

The method employed for the detection and isolation of plasmid DNA was the same as that in the previous paper<sup>8)</sup> except that half strength ZoBELL medium instead of TSB medium was used for the cultivation of strains.

#### Preparation and Fusion of Protoplast

For the preparation of protoplasts, mycelia of strain SS-939 (Trp<sup>-</sup>) and SS-1507 (Met<sup>-</sup>) were grown in half strength ZOBELL medium containing 0.5% (w/v) glycine for 1~2 days at 27°C. The mycelia were centrifuged at 3,000 × *g* for 10 minutes, washed twice with P medium,<sup>10)</sup> and suspended in P medium containing 1.5 mg/ml lysozyme (Sigma). After incubation of the mycelia at 32°C for 60~90 minutes, the protoplast suspension was filtered through cotton wool. Subsequently, the suspension was filtered through Uni-Pore Polycarbonate Membrane filters with a 2.5-μm pore size (Bio-Rad Labo.). About 10<sup>8</sup>/ml of protoplast suspension of strains SS-939 and SS-1507 were mixed. The mixture was centrifuged at 1,000 × *g* for 10 minutes, and washed with 5 ml of P medium. The resultant supernatant was decanted, and the protoplasts were dispersed in the remaining volume of supernatant by gentle shaking. A small volume (0.1 ml) of this dense protoplast mixture (about 10<sup>9</sup>~10<sup>10</sup>/ml) was added to 0.9 ml of 40% (w/v) Polyethyleneglycol (PEG) 4,000 (Sigma) solution. After 1~2 minutes, the suspension was mixed with 9 ml of P medium and plated on R3 agar medium.<sup>11)</sup> Melted soft agar R3 medium (Low Gelling Temperature Agarose, Marine Colloids, Inc., Rockland, Me., U.S.A.) kept at 32°C was then mixed with protoplasts on the agar plates.<sup>12)</sup> The plates were incubated for 7~10 days at 27°C.

#### Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed using 0.7% (w/v) agarose (Seakem ME) slab gel (horizontal type) in TBE buffer system (90 mM Tris, 90 mM boric acid and 4 mM Na<sub>3</sub>-EDTA, pH 8.3). After electrophoresis, the gels were stained in 0.5 μg/ml ethidium bromide for 30 minutes and the DNA bands were visualized under UV light (254 nm).

#### Digestion of Plasmid DNA with Restriction Endonucleases

Restriction endonucleases were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) except for *Bcl*I which was purchased from Bethesda Research Laboratories Inc. (Rockville, Md., U.S.A.). All enzymes were used according to the manufacturers' recommendations.

#### Nick Translation and SOUTHERN Hybridization

Nick translation of plasmid DNA to obtain a <sup>32</sup>P-labelled DNA probe and subsequent hybridization to plasmids obtained from *S. tenjimariensis* strains were carried out as described by SOUTHERN<sup>13)</sup> and MANIATIS, *et al.*<sup>14)</sup>

### Results

#### Plasmids from *S. tenjimariensis*

We isolated seven strains of *S. tenjimariensis* showing the same phenotypes including istamycin production. When the plasmids from each strain were isolated and electrophoresed, three strains having different plasmid profiles were observed. These strains were SS-939, SS-980 and SS-1507 (Fig. 1-A). Plasmid profiles of the other strains examined were the same as that of strain SS-939. In addition, protoplast fusion between SS-939 and SS-1507 provided three clones having plasmid profiles different from the parental strains. Two strains, F9 and F12, harbored almost all plasmids of both parent strains. Strain F4, however, had a new plasmid not present in the parental strains and smaller than any plasmid observed in the parental strains. The other plasmids in strain F4 were identical to those of SS-939 (Fig. 1-A). Plasmids from strains SS-939, SS-980, SS-1507 and F4, except for the large plasmids, were designated as indicated in Fig. 1-B.

#### Endonuclease Digestion of pST Plasmids

Each plasmid was purified after electrophoresis by phenol/chloroform treatment and ethanol precipitation. The purified plasmids were digested with restriction endonucleases to analyze their cleavage sites and molecular sizes. There were no restriction sites for *Eco*R I and *Hind* III. Six of

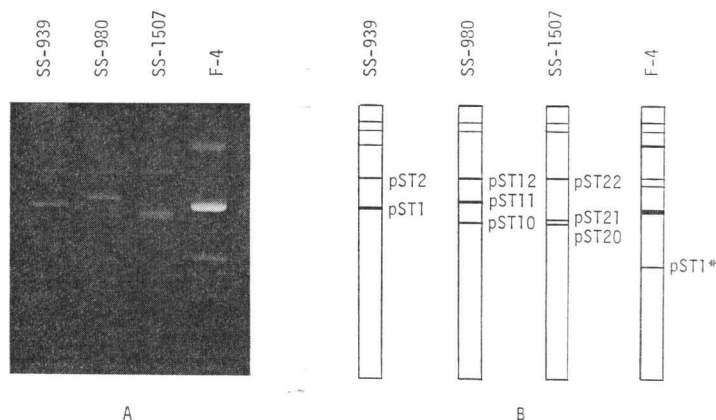
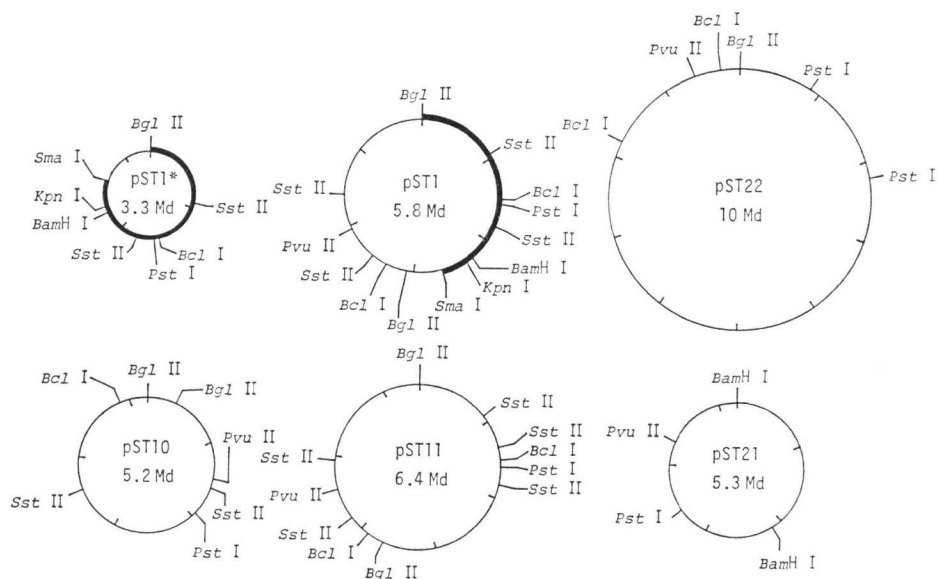
Fig. 1. Agarose gel electrophoresis of pST plasmids from *S. tenjimariensis*.

Fig. 2. Restriction endonuclease cleavage maps of pST plasmids.



the relatively small plasmids (3.3~6.4 Md), not including pST2, pST12 and pST22 (10 Md) were generally cut at 1 or 2 sites with *Bam*H I, *Bcl* I, *Bgl* II, *Kpn* I, *Pst* I and *Pvu* II. The three 10 Md plasmids differed from the smaller plasmids that there were more than 3 cleavage sites for *Bam*H I and more than 5 cleavage sites for *Sal* I and *Sst* II (Table 1).

#### Restriction Endonuclease Cleavage Maps of pST Plasmids

Cleavage maps of pST plasmids were constructed by double and/or triple restriction endonuclease digestions. The maps of six plasmids are shown in Fig. 2. When plasmids of each strain were compared with each other, no apparent similarity was recognized between plasmids harbored in each naturally-occurring strains. It was observed, however, that pST1\* in strain F4 had a homologous region from the *Bgl* II site to the *Sma* I site with pST1 (shown with thick line in Fig. 2). Homology or similarity was found between some plasmids harbored in different naturally-occurring strains, since

Table 1. Cleavage of plasmids by restriction endonucleases.

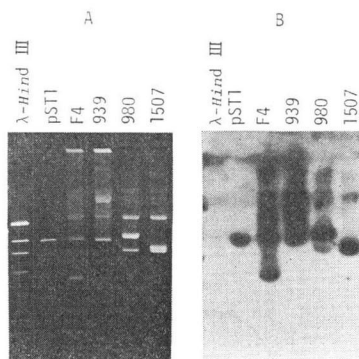
Strain	Plasmid	MW ( $\times 10^6$ )	Number of restriction sites									
			<i>Bam</i> H I	<i>Bcl</i> I	<i>Bgl</i> II	<i>Eco</i> R I	<i>Hind</i> III	<i>Kpn</i> I	<i>Pst</i> I	<i>Pvu</i> II	<i>Sal</i> I	<i>Sst</i> II
F4	pST1*	3.3	1	1	1	0	0	1	1	0	2	2
SS-939	pST1	5.8	1	2	2	0	0	1	1	1	3	4
F4	pST2	10				0	0	1	2	1	>5	>5
SS-980	pST10	5.2	0	1	2	0	0	0	1	1	3	2
	pST11	6.4	0	2	2	0	0	0	1	1	3	5
	pST12	10	>3	2	1	0	0	1	2	1	>5	>5
SS-1507	pST20	5.2	0	1	2	0	0	0	1	1	3	2
	pST21	5.3	2	1	0	0	0	3	1	1	2	2
	pST22	10	>3	2	1	0	0	1	2	1	>5	>5

Fig. 3. Interstrain comparison of pST plasmids from *S. tenjimariensis* by SOUTHERN blot hybridization using pST1 as a probe.

A: Agarose gel electrophoresis of plasmid DNA.

B: Autoradiograms of A.

Upper bands of lanes, F4, 939, 980 and 1507, in B correspond to the open circular plasmid DNA.



carried out to confirm the relationship observed in the restriction maps. As shown in Fig. 3,  $^{32}\text{P}$ -labelled pST1 hybridized strongly to pST1\*, pST1 and pST21 (and/or pST20), and slightly to pST10 although pST1 showed no clear similarity to pST10 and pST21 in the restriction maps. Similar results were obtained by hybridization analyses using  $^{32}\text{P}$ -labelled pST1\* and pST10 as the probes. On the other hand,  $^{32}\text{P}$ -labelled pST22 hybridized to pST2, pST12 and pST22 but not to the other plasmids (data not shown).

From the restriction map and SOUTHERN hybridization data, it was concluded that pST10 and pST20 were identical, that pST2, pST12 and pST22 were also identical, and that pST1\*, pST1 and pST11 were closely related to each other. pST21, which showed high homology by SOUTHERN hybridization, was not found to be related in the restriction maps.

### Discussion

We showed that three naturally-occurring strains of istamycin producing *S. tenjimariensis* have different plasmid profiles. Restriction endonuclease cleavage maps and SOUTHERN hybridization analyses of those plasmids revealed that there is homology among plasmids of different strains. A

pST10 of strain SS-980 and pST20 of strain SS-1507 showed the same restriction maps, as well as pST2 of strain SS-939, pST12 of strain SS-980 and pST22 of strain SS-1507 had identical restriction maps. Furthermore, pST1 of strain SS-939 had a high degree of homology with pST11 of strain SS-980, except that pST1 had a *Bam*H I-*Kpn* I region not present in pST11, and pST11 had an extra *Sst* II site between the *Sst* II and *Bcl* I sites in the *Sst* II-*Bcl* I-*Pst* I-*Sst* II region of pST1.

#### Hybridization among pST Plasmids

Nick translation of pST1\*, pST1, pST10 and pST22 to obtain  $^{32}\text{P}$ -labelled DNA probes and subsequent hybridization of pST plasmids from the four strains of *S. tenjimariensis* were

10 Md plasmid was common to all three strains. In addition, there were different plasmids smaller than 10 Md showing similarity to each other.

It seemed possible that the difference in plasmid profiles was due to spontaneous change of plasmids by subculturing these strains after isolation from soils. However, no change was observed after several subcultures of each strains. Therefore, it is probable that the difference in plasmid profiles among strains SS-939, SS-980 and SS-1507 preexisted in the natural environment, and that the small but clear differences between pST1 and pST11 occurred by DNA rearrangement in the natural environment. Evidence for plasmid variability was further obtained by protoplast fusion of strains SS-939 and SS-1507. Clone F4 harbored a small plasmid (pST1\*) which had a common region with the pST1 plasmid from strain SS-939. This indicates that the pST1 plasmid is changeable and pST1\* plasmid is probably the deletion derivative of pST1 plasmid. While DNA rearrangement within a plasmid is known to be provoked by "curing" treatment in certain species of *Streptomyces*,<sup>15)</sup> our observation in *S. tenjimariensis* strains will be the first case of plasmid DNA rearrangement in the natural environment. It seems likely that *S. tenjimariensis* SS-939, SS-980 and SS-1507 have descended from a common strain, and DNA rearrangement occurred during successive generations in the natural environment. This is concluded because they were isolated at the same location during three successive years, and they were identical in the taxonomical and physiological properties that were examined.

On the other hand, it seems unlikely that DNA rearrangement of the plasmids significantly influenced istamycin production because three strains of *S. tenjimariensis* produced similar amounts of istamycins. The function of the plasmids examined in this study is unknown as is the all function of all other cryptic *Streptomyces* plasmids except for SCPI.<sup>16)</sup> However, since the small plasmids of *S. tenjimariensis* strains were found to have only 1 or 2 sites for several restriction endonucleases it may be possible to construct plasmid cloning vector for gene manipulation in *S. tenjimariensis*. Among plasmids isolated, the pST1\* and pST1 will be the best candidates for vector development because they have the common portion necessary for replication and the non-homologous portion can be substituted with other foreign DNA fragment.

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