ISOLATION AND CHARACTERIZATION OF A POCK-FORMING PLASMID pTA4001 FROM *STREPTOMYCES LAVENDULAE*

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A covalently closed circular (ccc) DNA with a size of 5.9 kb was isolated from a strain producing streptothricins, *Streptomyces lavendulae* No. 1080, and its restriction map was determined. Colonies of the plasmid-carrying strain formed pocks on the lawn of the plasmid-free derivatives of the same organism. The pock-forming ability of pTA4001 was confirmed by transformation of *S. lividans* with the plasmid DNA. Insertion of the chromosomal streptothricin resistance gene of *S. lavendulae* No. 1080 into pTA4001 gave various composite plasmids with marked deletion in *S. lividans* as a host. By analyzing these derivatives, a 2.2 kb region essential for replication and a possible region for pock formation were determined in the map of pTA4001. A cloning vector, pKST2 (4.3 kb) containing resistance genes to streptothricin and thiostrepton with several unique cloning sites was constructed from pTA4001.

Since the first report on possible involvement of plasmid in antibiotic production[1] in streptomycetes, extensive screening for plasmids as well as studies on their biological functions in this practically important microorganisms have been conducted[2~14]. Among the plasmids isolated and physically characterized through these studies, only one plasmid, pSV1, has been proved to encode for antibiotic production and resistance (methylenomycin A)[15]. On the other hand, a characteristic biological function to form "pocks" has been ascribed to several *Streptomyces* plasmids[8,14]. These plasmids confer host cells a special conjugative ability which is detected by the formation of clear zone or "pocks" around colonies of the plasmid-bearing strain plated on the lawn of the plasmid-free strain. Exact mechanism of this phenomenon has not yet been elucidated, however, it is very useful in detection of transformants[16] with these plasmid DNAs. Several useful vectors containing selective antibiotic resistance markers for cloning in streptomycetes have been constructed from the pock-forming plasmids[14]. These hybrid plasmids facilitate the application of DNA cloning technique for genetic analysis and breedings in some species of streptomycetes. The *Streptomyces* includes so many species, however, that it is desirable to develope other vectors whose origins are different. For this reason, the screening of new plasmids and their physical and functional characterization remains important.

In this paper we describe the isolation and characterization of a new pock-forming plasmid pTA4001, with size of 5.9 kb, from *Streptomyces lavendulae* No. 1080, as well as the construction of new cloning vectors using this plasmid.

Materials and Methods

Bacterial Strains and Plasmids

S. lavendulae No. 1080 was isolated as a producer of streptothricins from soil. It produces the streptothricin group antibiotic(s) and melanine pigment. The strain forms abundant aerial mycelia

with spores and is resistant to streptothricins. The plasmid pTA4001 was found and isolated from this strain. *S. lividans* TK21 provided by D. A. HOPWOOD and *S. coelicolor* M132 provided by M. OKANISHI were used as hosts for recombinant plasmids.

pIJ41-SR51 was used as a source of the streptothricin resistance gene. It contains the resistance gene on a 1.6 kb DNA fragment of *S. lavendulae* No. 1080 inserted at the *Bam* HI site on pIJ41. Construction of the hybrid plasmid will be described elsewhere. pIJ303[14] constructed and provided by D. A. HOPWOOD was used as a source of the thiostrepton resistance gene.

Media, Reagents and Enzymes

NYM medium contained nutrient broth 2%, yeast extract 0.2%, NaCl 0.8%, maltose 0.5%, pH 7.2. MB medium contained maltose 1%, nutrient broth 0.3%, yeast extract 0.1%, pH 7.2. YEME medium described by BIBB *et al.*[3] contained yeast extract 0.3%, Bacto peptone 0.5%, malt extract 0.3%, glucose 1%. Media for protoplasting and regeneration of *S. lividans* in transformation experiments were the same as described by OKANISHI *et al.*[17].

Streptothricin mixture and thiostrepton were obtained from Asahi Kasei Co. Ltd. Restriction enzymes were purchased from New England BioLab, BRL or Takara Shuzo Co. Ltd.

Preparation of Plasmid DNA

In order to obtain mycelia of *S. lavendulae* No. 1080, a 50-ml seed culture in NYM medium was inoculated into 500 ml of NYM medium containing 0.5% glycine in a 5-liter Erlenmeyer flask and cultured aerobically with rotally shaking at 130 rpm for 3 days at 26.5°C. The mycelia were harvested by centrifugation and washed twice with buffer containing 50 mM Tris-HCl, 20 mM EDTA, pH 8.0. Washed mycelia were suspended in 25 ml of buffer containing 25% (w/v) sucrose, 30 mM Tris-HCl, 20 mM EDTA, pH 8.0, and 5 ml of 30 mg/ml lysozyme and 10 ml of 0.25 M EDTA (pH 8.0) were added to the suspension. After the incubation at 37°C for 30 minutes, cells were solubilized by addition of 5 ml of 20% SDS. To the lysate 12.5 ml of 5 M NaCl was added and the mixture was stored at 0°C for more than 3 hours. The cleared lysate was obtained by ultracentrifugation at 48,000×*g* for 1 hour. After the treatment with equal volume of phenol saturated with water, DNA was precipitated by addition of ethanol and the precipitated DNA was loaded on CsCl-ethidium bromide (EtBr) density gradient centrifugation. ccc DNA was isolated from the lower band.

In the case of *S. lividans*, YEME medium was used for cultivation, phenol treatment was omitted and DNA was precipitated by polyethyleneglycol 6000 addition.

Electron Microscopy

pTA4001 sample was prepared for electron microscopy by the formamide method[18] and viewed with a Hitachi HU12 electron microscope.

Restriction Enzyme Digestion and Agarose Gel Electrophoresis

For restriction enzyme digestion, DNA was digested with each enzyme for 2 hours under the conditions according to the supplier's instructions. Agarose gel electrophoresis was as in SHARP *et al.*[19]. *Hin*d III digests of phage lambda and *Hae* III digests of pBR322 were used as molecular size markers.

DNA Ligation and Transformation

Each DNA fragment generated by restriction enzyme digestion was ligated to each other with T4 DNA ligase at 22°C for 2 hours.

Preparation of protoplasts and transformation were performed according to the method of BIBB *et al.*[16].

Results

Isolation and Restriction Analysis of the Plasmid pTA4001

The ccc DNA was detected and isolated by the CsCl-EtBr density gradient centrifugation from the cleared lysate of the streptothricins-producing *S. lavendulae* No. 1080. This plasmid was designated pTA4001. Its size was determined to be 5.9 kb by electron microscopy (Fig. 1) which was consistent with that estimated by agarose gel electrophoresis.

Fig. 1. Electron micrograph of the plasmid pTA4001.
The bar under the photograph indicates the length of 1 μm.

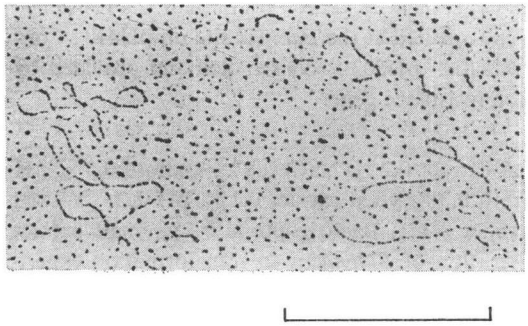


Fig. 2. Restriction enzyme map for pTA4001.
Rep. indicates the 2.2 kb region in which the gene essential for replication of this plasmid is involved.

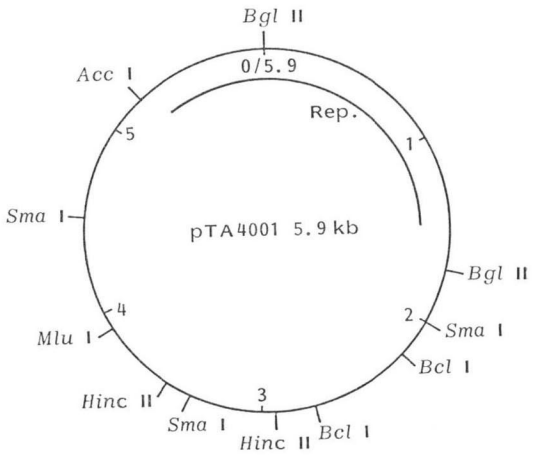


Table 1. Restriction patterns of pTA4001.
In the parentheses the molecular sizes of each generated fragments (kb) are described.

No. of cleavage sites	Enzymes
1	*Acc* I, *Mlu* I
2	*Bcl* I (5.4, 0.5)
	Bgl II (4.2, 1.7)
	Hinc II (5.3, 0.6)
3	*Sma* I (3.4, 1.4, 1.1)
Many	*Alu* I, *Eco* RII, *Hae* II
	Hae III, *Hha* I, *Hpa* II
	Mbo I, *Taq* I
0	*Bam* HI, *Eco* RI, *Hind* III
	Hpa I, *Kpn* I, *Pst* I
	Sal I, *Xba* I, *Xho* I

pTA4001 was analyzed with various restriction endonucleases. *Bgl* II, *Bcl* I, *Hinc* II, *Acc* I, *Mlu* I and *Sma* I cleaved pTA4001 at sites of 2, 2, 2, 1, 1 and 3, respectively. The restriction patterns are shown in Table 1. According to cleavage analysis with combined use of the restriction enzymes, the restriction map was constructed as shown in Fig. 2.

Pock-forming Ability of pTA4001

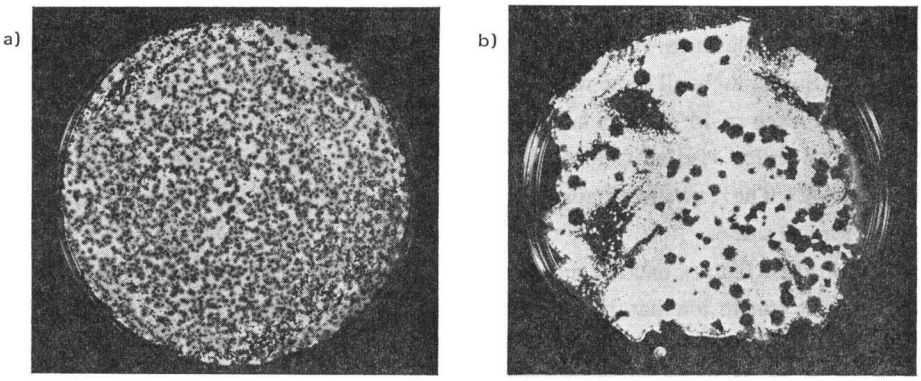
In order to know the biological functions of pTA4001 in *S. lavendulae* No. 1080, curing treatments with acridine orange, acriflavine or cultivation at high temperature were carried out. By the treatment with acridine orange (10 μg/ml), many mutants deficient for streptothricins and melanine production, aerial mycelium formation and streptothricin resistance were obtained at high frequency (2~10%), however, no loss of pTA4001 was observed in these mutants. On the other hand, a streptothricins-non-producing mutant, TN31, obtained with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment (500 μg/ml) was found to lack pTA4001. This mutant showed decreased resistance to streptothricins (below 5 μg/ml), however, direct relationship between loss of the plasmid and the decreased resistance could not be established.

On the other hand, when a streptothricin-non-producing mutant still carrying pTA4001, TN35, grew on the lawn of the plasmid-free mutant, TN31, pock formation was observed. These pocks were very small and sometimes invisible, however, this phenomenon suggests that pTA4001 has pock-forming ability. Pock formation by TN35 was also observed on the lawn of *S. lividans* TK21 (Fig. 3a). In this case, the pock formation was clear and the efficiency per TN35 inoculated was nearly 100%. This result suggests the conjugation between *S. lavendulae* and *S. lividans*, however, it is necessary to investigate futher on this suggestion.

Fig. 3. Pock formation by pTA4001.

a) Pocks formed by pTA4001-carrying strain, *S. lavendulae* TN35, on the lawn of pTA4001 non-carrying strain, *S. lividans* TK21.

b) Pocks formed by transformation of pTA4001 into *S. lividans* TK21.



In order to confirm the pock-forming ability of pTA4001, we attempted to introduce pTA4001 DNA into *S. lividans* TK21 by transformation. After the transformation with the standard procedure, very large pocks appeared in the lawn on the regeneration plates and the spore formation in the pocks was considerably delayed (Fig. 3b). This result clearly shows that pTA4001 has pock forming ability.

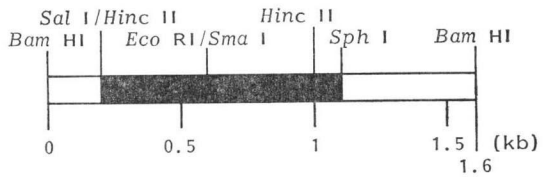
In order to confirm that pTA4001 was compatible with pIJ303 and pIJ41-SR51, *S. lividans* TK21 was transformed by using the DNA mixture of pTA4001 and very low amounts of pIJ303 and pIJ41-SR51. Among the resultant transformants which showed both thiostrepton and streptothricin resistance, one strain was found to contain not only pIJ303 and pIJ41-SR51 but also pTA4001. This indicates pTA4001 is compatible with pIJ101 and SLP1.2 which are the original plasmids of pIJ303 and pIJ41-SR51, respectively. The intensity of fluorescence of pTA4001 band in agarose gel was about half as much as that of pIJ303 (40~300 copies per chromosome) and extremely higher than that of pIJ41-SR51 (3~4 copies per chromosome). From this observation, the copy number of pTA4001 in *S. lividans* TK21 was estimated to be relatively high.

Construction of PTA4001 Variants and Functional Analysis

In order to determine the regions coding for the genes essential for replication and those for pock formation, derivative plasmids were constructed by inserting the streptothricin resistance gene into pTA-4001. This gene had been cloned from chromosomal DNA of *S. lavendulae* No. 1080 into *S. lividans* by using pIJ41 as a vector. The resulting hybrid plasmid pIJ41-SR51 had this gene within a 1.6 kb *Bam* HI fragment (Fig. 4). This fragment was purified from *Bam* HI digested pIJ41-SR51 by agarose gel electrophoresis. The 1.6 kb *Bam* HI fragment was ligated with linear DNA fragments obtained from pTA4001 completely digested with *Bgl* II or *Bcl* I, and the ligation mixture was introduced into *S. lividans* protoplasts by transformation. Spores on the regeneration plate were replicated to MB plate containing 20 μg/ml streptothricins. No transformants were obtained from the experiment using the *Bgl* II digested pTA4001, while 14

Fig. 4. Physical map of the 1.6 kb *Bam* HI fragment encoding streptothricin resistance gene.

The gene have been proved to exist within the region indicated as solid box.



transformants were detected from the experiment using *Bcl* I digested pTA4001. But these transformants were all deficient for the ability of pock formation. These results suggest that one of the *Bgl* II sites is associated with the region essential for the replication of this plasmid and *Bcl* I sites for pock formation.

The recombinant plasmids were isolated from these transformants and analysed with restriction endonucleases. Surprisingly all these recombinant plasmids were different in molecular sizes from the expected ones. Eleven of 14 recombinant plasmids clearly showed that *in vivo* deletion had occurred, while 3 seemed to suffer deletion following the multiple ligation between the vectors and the 1.6 kb *Bam* HI fragments. The restriction maps and deletion sites in several of these recombinants, pKS series, are shown in Figs. 5 and 6.

As shown in these figures, one of the *Bgl* II sites of pTA4001 is conserved in all cases. This result supports the former conclusion that the conserved *Bgl* II site is essential for replication of these plasmids. Moreover Fig. 6 indicates that the gene(s) essential for replication exists within a 2.2 kb region contain-

Fig. 5. Restriction enzyme maps for pKS series plasmids.

The maps are represented in the linear forms with the zero point chosen at the conserved *Bgl* II site. Abbreviations for restriction enzymes include *Bgl* II (Bg), *Sma* I (Sm), *Bcl* I (Bc), *Mlu* I (Ml), *Eco* RI (Ec) and *Sal* I (Sa).

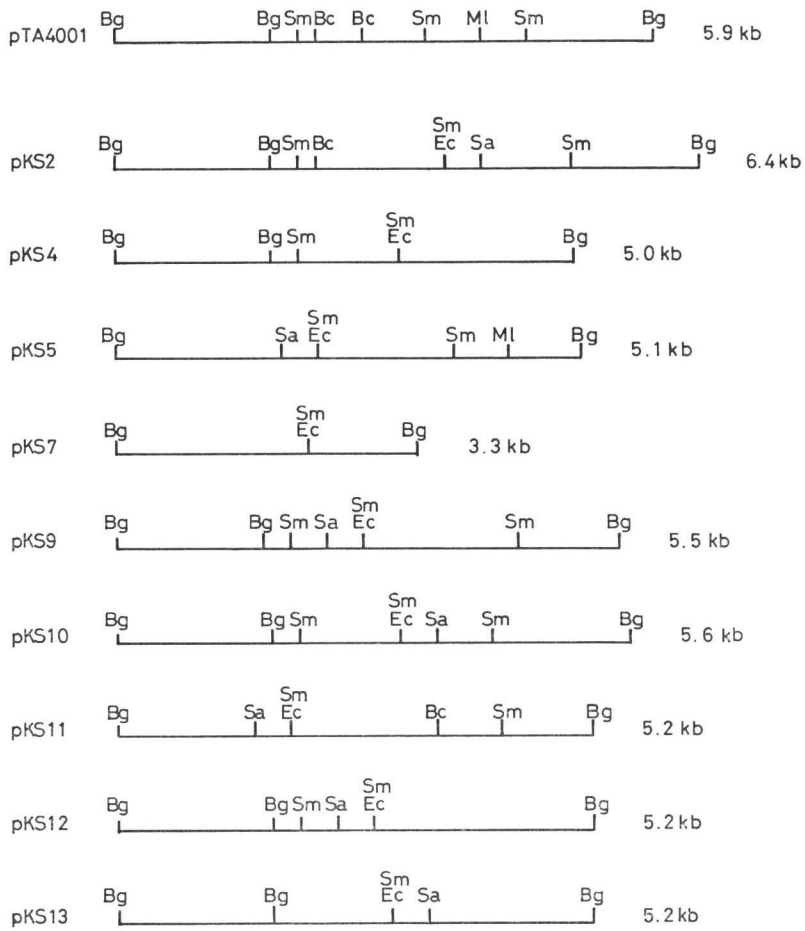
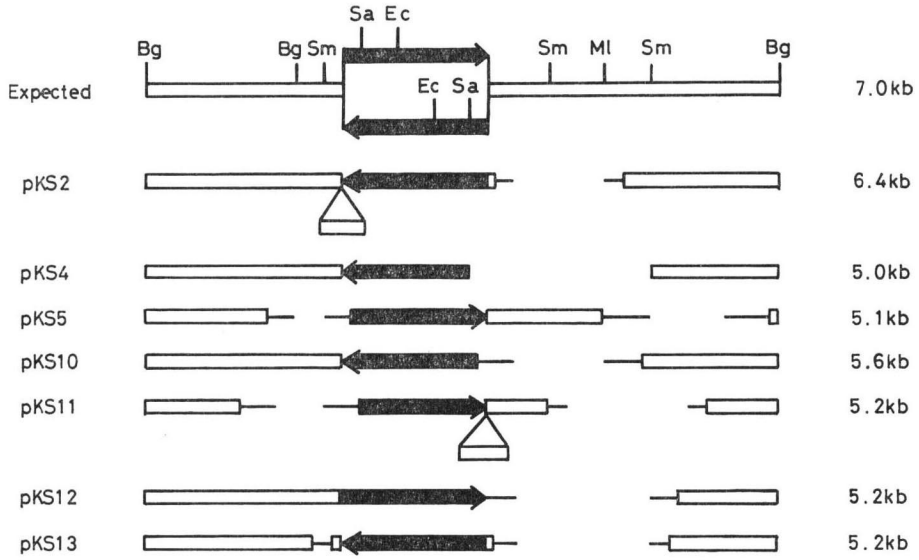


Fig. 6. Deletion sites of pKS series plasmids.

The heavy arrow shows the position and direction of the DNA fragment carrying the resistance gene to streptothricin. But the direction does not indicate that of transcription. The insertion fragment of pKS2 and pKS11 are 0.5 kb *Bcl* I fragments derived from pTA4001. Narrow line indicates the region in which the deletion end exists. Abbreviations are same with Fig. 5.



ing the conserved *Bgl* II site of PTA4001.

All the pKS series are deficient for pock formation. Minimum deletion causing this deficiency is observed with pKS2. At least a part of the pock-forming genes might be present within this region.

In order to know whether pKS series originated from pTA4001 can replicate in *S. coelicolor*, *S. coelicolor* M132 was transformed with pKS2, pKS7 and pKS11. From the transformants showing streptothricin resistance, the plasmid DNAs were isolated by the method of THOMPSON *et al.*[20]. These plasmids were compared with original pKS2, pKS7 and pKS11 by agarose gel electrophoresis, which showed the identical molecular sizes and restriction patterns with those of the original plasmids.

Construction of New Cloning Vectors

In order to construct the useful cloning vectors, the smallest plasmid in pKS series, pKS7 (3.3 kb), which contained a deleted 1.6 kb fragment coding streptothricin resistance gene, was improved by inserting thiostrepton resistance gene from pIJ303. This gene cloned by HOPWOOD *et al.* from *S. aureus* locates in a 1.0 kb *Bcl* I fragment of pIJ303.

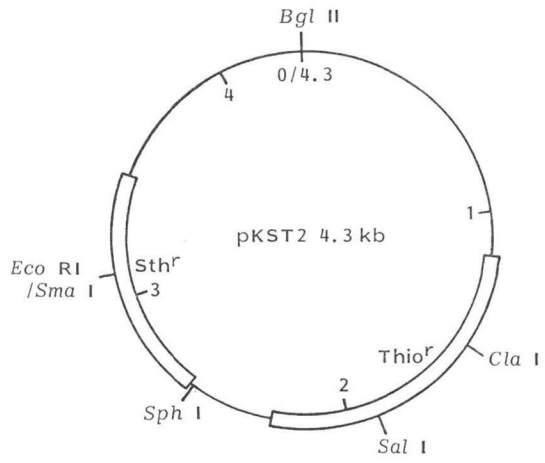
pKS7 has each a single site for *Bgl* II, *Eco* RI, *Sma* I and *Sph* I cleavage. In order to keep most of these unique cloning sites in the composite plasmids, we intended to insert the thiostrepton resistance gene into one of the *Mbo* I sites which exist more than 10 in pKS7. pKS7 DNA was treated with very low amount of *Mbo* I and the resultant partial digests were ligated with the 1.0 kb *Bcl* I fragment containing the thiostrepton resistance gene. The ligation mixture was introduced into *S. lividans* TK21 and transformants were selected by replicating the spores on regeneration plates to MB agar medium containing 40 μg/ml thiostrepton and 20 μg/ml streptothricins.

The recombinant plasmids, pKST series, which had the streptothricin and thiostrepton resistance genes had variety in the *Mbo* I sites of pKS7 in which the *Bcl* I fragment was inserted. Restriction map

of pKST2 (4.3 kb), one of these plasmids, are shown in Fig. 7. *Sal* I, *Cla* I, *Eco* RI, *Sma* I and *Sph* I sites of pKST2 are possible cloning sites. Among them, the *Sal* I and *Cla* I sites within thiostrepton resistance gene and perhaps the *Eco* RI and *Sma* I sites within streptothricin resistance gene allow selection of clones according to inactivation of these resistance genes by insertion of foreign DNA.

In order to know the host range, pKST2 was introduced into various streptomycetes by transformation. Streptothricin and thiostrepton resistant transformants were obtained from the experiments using *S. coelicolor* M132, *S. griseus* IFO13189, *S. viridochromogenes* 6A36 and *S. lavendulae* TN31, suggesting pKST2 had relatively wide host range.

Fig. 7. Restriction enzyme map of pKST2.
Double line segments represent the resistance genes to streptothricin (Sth^r) and thiostrepton (Thio^r).



Discussion

A strain deficient for plasmid pTA4001 was derived by the NTG treatment from *S. lavendulae* No. 1080. It showed lack of streptothricin production and resistance but still retained melanine productivity and ability to form aerial mycelia. On the other hand, "curing" treatment of the organism with acridine orange produced mutants deficient for productivities of streptothricins and melanine, resistance to streptothricins and formation of aerial mycelia with high frequencies, however, all these mutants were found to contain the plasmid pTA4001. Some plasmid other than pTA4001 or a transposable genetic determinant may be involved in these biological activities of *S. lavendulae* No. 1080. Another plasmid pSL1 with size of 3.9 kb has been reported from a strain of *S. lavendulae*[21], however, it seems not to encode any apparent biological functions including pock formation.

pTA4001 can form pocks on the plasmid-free strain of *S. lavendulae* and *S. lividans*, and pKST2 originated from pTA4001 can replicate in *S. lividans*, *S. coelicolor*, *S. griseus*, *S. viridochromogenes* and *S. lavendulae*. These results suggest that pTA4001 is a conjugative plasmid with relatively wide host range. Among several conjugative plasmids found from streptomycetes, pTA4001 has the smallest molecular size of only 5.9 kb. The derivatives of pTA4001 carrying the streptothricin resistance gene could be introduced into *S. lividans* harvoring pIJ41-SR51 and pIJ303 which were derived from SLP1.2 and pIJ101, respectively. Thus pTA4001 seems to be a different replicon from them and is expected to have a different host range.

Insertion of the streptothricin resistance gene into this plasmid gave various deletion plasmids in *S. lividans*, the smallest of which was pKS7 with size of 3.3 kb. pKST2 (4.3 kb) constructed from pKS7 has two resistance genes to streptothricin and thiostrepton which allow negative selection for cloning at several unique restriction sites. It is stably retained in *S. lividans* as a host with relatively high copy numbers. From these characteristics, pKST2 is potentially a useful cloning vector for streptomycetes.

Acknowledgment

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