CLONING AND CHARACTERIZATION OF THE STREPTOTHRICIN-RESISTANCE GENE WHICH ENCODES STREPTOTHRICIN ACETYLTRANSFERASE FROM *STREPTOMYCES LAVENDULAE*

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The streptothricin-resistance gene of *Streptomyces lavendulae* No. 1080 was cloned in *S. lividans* using pIJ41 as a vector. From subcloning experiments, the 1.6 kb *Bam*H I fragment was determined to encode the structural gene. The cell extracts of *S. lividans* carrying the gene on the plasmid pKS7 had activity to inactivate streptothricin in the presence of *S*-acetyl coenzyme A, indicating that the gene product was streptothricin acetyltransferase.

A large number of antibacterial antibiotics are produced by strains of *Streptomyces* and their relatives, which possess self-resistance genes and mechanisms to the antibiotics they produce. The recent development of host-vector systems for *Streptomyces* has enabled the cloning of several of these genes encoding resistance to neomycin[1], thiostrepton[1], viomycin[2], ribostamycin[3] and streptomycin[4]. Such studies will throw light on the possible relationship between the resistance genes of antibiotic-producing microorganisms and those from various bacteria, which frequently are carried on a drug-resistance plasmid. It is also expected that this will provide new selective markers useful for molecular cloning in *Streptomyces* sp.

The streptothricin group antibiotics produced by various *Streptomyces* strains are potent inhibitors of prokaryotic protein biosynthesis and have a broad antibacterial spectrum, but their characteristic delayed toxicity has prevented clinical application. Little is known about the mechanisms of resistance to these antibiotics although it may be relevant that *N*-acetylation of the β-lysine moiety of one of these antibiotics caused loss of antibacterial activity[5], and that acetylstreptothricin was found from a *Streptomyces* strain as an antitumor agent[6]. We isolated a streptothricin-producing strain No. 1080 of *Streptomyces lavendulae* which harbored a small pock-forming plasmid pTA4001[7]. In this paper, we describe cloning of a chromosomal streptothricin-resistance gene (Sth^r) of *S. lavendulae* No. 1080 as well as detection of streptothricin acetyltransferase activity encoded by the cloned gene.

Materials and Methods

Bacterial Strains and Plasmids

S. lavendulae No. 1080 isolated from soil as a streptothricin producer was used as a donor of the resistance gene. A host-vector system, *Streptomyces lividans* TK21 and pIJ41, kindly provided by D. A. HOPWOOD, was used for cloning. pTA4001, a pock-forming, multi-copy plasmid found in *S. lavendulae* No. 1080[7] was also used to construct hybrid plasmids carrying the Sth^r gene. *Escherichia coli* HB101 and pBR322 were also used for sub-cloning. *Bacillus subtilis* ATCC 6633 was used as an indicator strain for streptothricin activity.

Media

MB medium containing nutrient broth 0.3%, yeast extract 0.1% and maltose 1%, pH 7.2, was used for cultivation of *Streptomyces* strains on plates. NYM medium containing nutrient broth 2%,

yeast extract 0.2%, NaCl 0.8% and maltose 0.5%, pH 7.2, was used to obtain *Streptomyces* mycelia for DNA preparation. Media for protoplasting and regeneration of *S. lividans* in transformation experiments were the same as described by OKANISHI *et al.*[8]. In the case of enzyme assay, cells were grown in CE medium containing Casamino Acids 0.5%, yeast extract 0.25%, $NaNO_3$ 0.3%, KH_2PO_4 0.1%, KCl 0.05% and $CaCO_3$ 0.5%.

Preparation of DNA

To obtain mycelia of *S. lavendulae* No. 1080, a 10-ml seed culture in NYM medium was inoculated into 100 ml of NYM medium containing glycine 0.5% and cultured aerobically with reciprocal shaking for 2 days at 26.5°C. The mycelia were harvested by centrifugation and washed twice with the buffer containing 50 mM Tris-HCl, 20 mM EDTA, pH 8.0. Washed mycelia were resuspended in 15 ml of buffer containing 25% sucrose (w/v), 50 mM Tris-HCl, 20 mM EDTA, pH 8.0, and 3 ml of 30 mg/ml lysozyme and 6 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 3 ml of 20% sodium lauryl sulfate followed by incubation at 55°C for 5 minutes. The lysate was diluted with equal volume of H_2O and then treated with equal volume of phenol saturated with H_2O, and DNA was precipitated by EtOH addition. Precipitated DNA was dissolved in a buffer containing 10 mM Tris-HCl, 1 mM EDTA, pH 7.8, and further purified by CsCl - ethidium bromide density gradient centrifugation. Chromosomal DNA was isolated from the upper band.

Preparation of plasmid DNA was carried out as described previously[7].

Restriction Enzyme Digestion, DNA Ligation and Transformation

DNA was digested with various restriction enzymes according to the supplier's instructions. Ligation was performed with T4 DNA ligase under the standard condition. Preparation of protoplasts and transformation were performed according to the method of BIBB *et al.*[9]. Agarose gel electrophoresis was used for analysis of DNA fragments and plasmids.

Nick Translation and Hybridization of DNA Digests

DNA was labeled by nick translation, using the nick translation kit purchased from Amersham Co., Ltd. The hybridization technique used was essentially the same as described previously[10].

Preparation of Crude Extract

Cells grown in CE medium were harvested and washed in 10 mM Tris-HCl (pH 7.2). Washed cells were frozen at −20°C and broken by grinding on ice with alumina. A buffer containing 10 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 10 mM NH_4Cl, 5 mM 2-mercaptoethanol was added and the alumina was removed by centrifugation at 10,000 × *g* for 1 hour at 4°C.

Bioassay for Streptothricin-inactivating Enzyme

One ml of reaction mixture containing 20 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 0.2 mg/ml streptothricin, 1 mM ATP or 0.34 mM *S*-acetyl coenzyme A and crude enzyme was incubated at 30°C for 1 hour, then 50 μl of this mixture was loaded on 8-mm paper disc. The disc was placed on the nutrient broth plate overlayed by soft agar containing *B. subtilis* ATCC 6633. Streptothricin-inactivating activity was determined by measuring the diameter of inhibitory zone.

Enzymes and Chemicals

Restriction enzymes and T4 DNA ligase were purchased from New England BioLab., BRL or Takara Shuzo Co., Ltd. Streptothricin and thiostrepton (Thio) were provided by Asahi Kasei Co., Ltd.

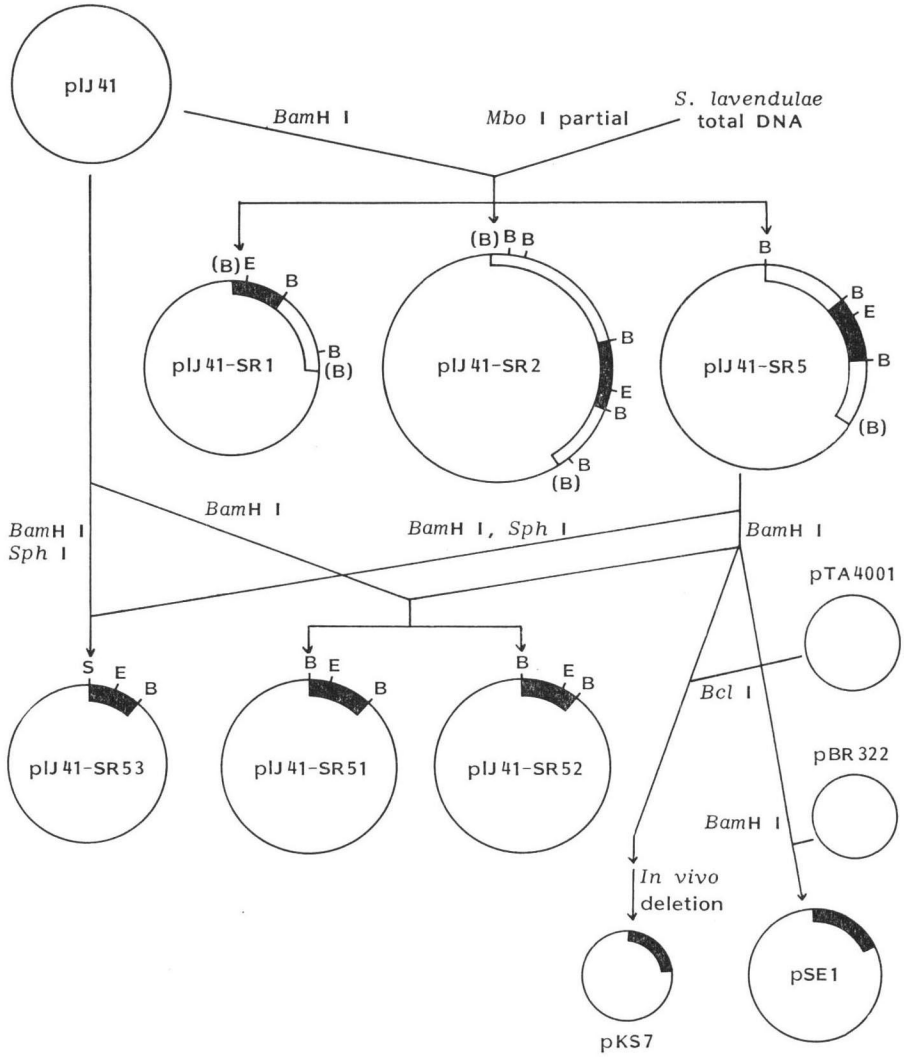
Results

Cloning of the Sth^r Gene from *S. lavendulae* No. 1080

S. lavendulae No. 1080 showed marked resistance to streptothricin with a 40 μg/ml MIC for growth, while that of *S. lividans* TK21 was less than 5 μg/ml. Chromosomal DNA of the former was partially digested with *Mbo* I and the resulting fragments were inserted into the unique *Bam*H I site

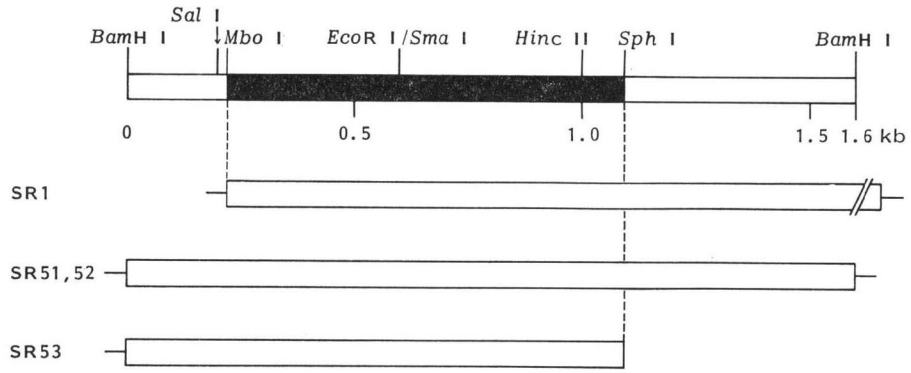
Fig. 1. Construction of recombinant plasmids carrying Sth^r gene.

The boxes represent the DNA fragments derived from *S. lavendulae*. The solid boxes are the common *Bam*H I fragments and its derivative. The abbreviations B, E and S indicate *Bam*H I, *Eco*R I and *Sph* I sites, respectively.



in pIJ41 which was located within neomycin-resistance gene. The ligation mixture was used to transform protoplasts of *S. lividans* TK21. After regeneration of the protoplasts, the transformants were selected by replicating the spores on the regeneration plates onto MB plates containing 20 μg/ml streptothricin and 40 μg/ml thiostrepton. Three Thio^r, Sth^r transformants were obtained each of which harbored different plasmids larger than pIJ41. Reintroduction of the recovered plasmids into the host gave Thio^r, Sth^r transformants having a resistance to streptothricin of more than 80 μg/ml. Restriction endonuclease digestion of these plasmids pIJ41-SR1, -SR2 and -SR5 revealed the presence of the insertion fragments of 3.8, 8.8 and 5.1 kb, respectively (Fig. 1). An identical *Bam*H I region (1.6 kb) with single *Eco*R I and *Sal* I site was observed in pIJ41-SR2 and -SR5. pIJ41-SR1 was identified to contain a large part of the same region which was generated by cleavage at a *Mbo* I site located

Fig. 2. Restriction map of the 1.6 kb *Bam*H I fragment and identification of Sth^r gene.
Three open bars represent the DNA regions which are carried on pIJ41-SR1, -SR51 or 52 and -SR53, respectively and the solid bar represents the common DNA region between these plasmids.



near the *Sal* I site (Fig. 2). No further homology was observed within the inserted DNAs probably due to tandem insertion of the different *Mbo* I fragments.

Sub-cloning of Sth^r Gene

The common 1.6 kb *Bam*H I region was isolated from pIJ41-SR5 and introduced into the *Bam*H I site of pIJ41 by using *S. lividans* as a host. Two types of the hybrid plasmids carrying the *Bam*H I fragment with an opposite orientation, pIJ41-SR51 and -SR52, were obtained (Fig. 1), both of which conferred Sth^r on the host. Analysis of the cloned *Bam*H I fragment gave the more detailed restriction map shown in Fig. 2. Further sub-cloning of a *Bam*H I-*Sph* I fragment (1.1 kb) of the region was performed by ligation of the fragment with *Bam*H I, *Sph* I double digested pIJ41. The resulting hybrid plasmid pIJ41-SR53 also expressed resistance to streptothricin of more than 80 μg/ml. These results indicate that the promoter and coding region for the Sth^r gene probably are located within the 1.6 kb *Bam*H I fragment and at least 0.9 kb of the *Mbo* I-*Sph* I fragment is sufficient to code for the amino acid sequence of the gene product (Fig. 2).

Two other hybrid plasmids were constructed by using the 1.6 kb *Bam*H I fragment, *i.e.*, pKS7[7] which was generated by ligation of the fragment with *Bcl* I digested pTA4001 followed by *in vivo* deletion in *S. lividans* to cause the loss of pock-forming ability of pTA4001, and pSE1 carrying the fragment at *Bam*H I site of pBR322 in *E. coli*. These plasmids were used in the following experiments.

Hybridization of the Cloned Sth^r Gene with Chromosomal DNA of *S. lavendulae*

In order to confirm the origin of the cloned Sth^r gene, southern hybridization of the 1.6 kb *Bam*H I fragment was performed with *Bam*H I digested chromosomal DNA of *S. lavendulae* No. 1080. In this experiment, the hybrid plasmid pSE1 carrying the Sth^r *Bam*H I fragment on pBR322 was used as a radioactive probe. As shown in Fig. 3, radioactively labeled pSE1 hybridized with a single *Bam*H I fragment of *S. lavendulae* chromosomal DNA but not with that of *S. coelicolor*. These results indicate that the cloned Sth^r gene came from chromosomal DNA of *S. lavendulae*. The slight difference in size between the hybridized band of *S. lavendulae* chromosomal DNA and that of pIJ41-SR51 was probably because the digested chromosomal DNA migrated differently from the plasmid or because at least one of the *Bam*H I sites observed in pIJ41-SR2 and -SR5 was not the original site but that generated secondarily by tandem insertion of different *Mbo* I fragments into a vector.

Fig. 3. Homology between the cloned *BamH* I fragment and total DNAs.

DNA patterns on agarose (1%) gel electrophoresis (left) and corresponding autoradiograms using ^{32}P-labeled pSE1 as a probe are shown (right).

Lane 1, 4, *S. coelicolor* A3(2) total DNA (3 μg); lane 2, 5, *Bam*H I digested total DNA of *S. lavendulae* No. 1080 (3 μg); lane 3, 6, *Bam*H I digested pIJ41-SR51 (0.1 μg).

The total DNA of *S. lavendulae* was digested with *Bam*H I at 37°C for overnight and the gel electrophoresis was performed at 60 volts in a Tris-acetate buffer.

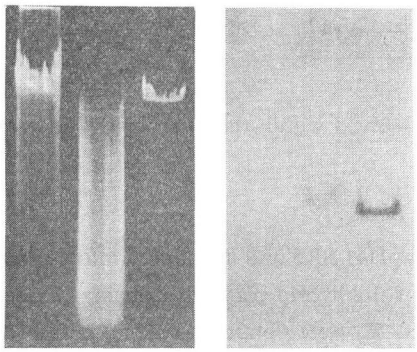
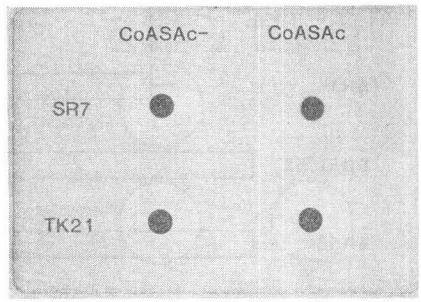


Fig. 4. Detection of *S*-acetyl coenzyme A dependent streptothricin-inactivating activity.

The activity of cell free extracts of *S. lividans* SR7 (carrying pKS7) and TK21 (plasmid non-carrying strain) were assayed as described in Materials and Methods in the absence (CoASAc−) and presence (CoASAc) of *S*-acetyl coenzyme A.



Detection of Streptothricin Acetyltransferase Activity Encoded by the Sth^r Gene

Inactivation of an antibiotic is one of the main resistance mechanisms in antibiotic-producing microorganisms. In order to know whether the streptothricin-inactivating enzyme was encoded by the cloned Sth^r gene, a cell-free extract of *S. lividans* harboring pKS7 (multi-copy plasmid constructed from Sth^r *Bam*H I fragment and pTA4001) was prepared and its inactivating activity was assayed in the presence of *S*-acetyl coenzyme A or ATP. As shown in Fig. 4, the inhibitory zone due to streptothricin disappeared in the presence of *S*-acetyl coenzyme A, while no inactivation occurred in the presence of ATP. These results imply that the Sth^r gene encodes streptothricin acetyltransferase.

Discussion

The cloned DNA fragment of *S. lavendulae* on the low copy vector pIJ41 conferred streptothricin-resistance to *S. lividans* which was equivalent to or even higher than that of the original streptothricin producer. In the case of neomycin-resistance, two resistance genes coding for neomycin acetyltransferase and neomycin phosphotransferase were cloned from *S. fradiae*. Introduction of one of these genes on a low copy vector into *S. lividans* was not sufficient to confer the high level of resistance as observed with the neomycin producer, for which simultaneous presence of the both genes were required[2]. High resistance by the cloned Sth^r gene indicates that the gene is involved in the major resistance mechanism in the streptothricin-producing organism.

The mechanism of streptothricin-resistance has never been elucidated, however, the presence of acetylstreptothricin among the products of streptomycetes[6] and inactivation of streptothricin by chemical acetylation of the free NH_2 of its β-lysine moiety[5] suggest that enzymatic inactivation by acetylation might be a possible resistance mechanism. This is supported by detection of a distinct acetyltransferase activity directed by the cloned Sth^r gene here reported. The maximum size for the coding region of the acetyltransferase was estimated to be 0.9 kb which corresponds to a molecular weight of approximately 26,000. Purification and identification of the enzyme will be reported in a subsequent paper.

The cloned Sth^r gene possesses single *Eco*R I and *Sma* I site within the coding region, which should allow inactivation by insertion of foreign DNA fragments at these sites. Also the broad and potent antibacterial activity of streptothricin makes the Sth^r gene a powerful selective marker for cloning. Since streptothricin has never been used for clinical and other practical purposes, application of the gene for cloning will have some advantage to avoid appearance in the environment of microorganisms resistant to the clinically useful antibiotics.

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