

CLONING OF STREPTOMYCIN  
RESISTANCE GENE FROM A  
STREPTOMYCIN PRODUCING  
STREPTOMYCETE

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Cloning of antibiotic resistance genes from antibiotic-producing organisms is important for recombinant DNA technology of antibiotic-producing organisms, since the resistance is useful as a selective marker of cloning vectors. Among known antibiotic producers, the resistance genes of neomycin<sup>1)</sup>, viomycin<sup>1)</sup>, methylenomycin<sup>2)</sup>, thiostrepton<sup>1)</sup>, erythromycin<sup>1)</sup>, ribostamycin<sup>3)</sup>, kanamycin<sup>3,4)</sup>, novobiocin<sup>3)</sup>, destomycin<sup>3)</sup> and racemomycin<sup>3)</sup> already have been cloned. Since the streptomycin (SM) resistance gene has not been cloned yet, we have cloned a SM resistance determinant which encodes SM 6-O-phosphotransferase from a SM producer to facilitate the development of host-vector system with SM resistance as a selective marker.

The donor strain was *S. griseus* ISP 5236, a SM producer. The recipient strain as a host for recombinant plasmids was *S. lividans* 1326 which was kindly supplied by Dr. C. THOMPSON.

Growth conditions and preparation of chromosomal and plasmid DNA were those described by CHATER *et al.*<sup>5)</sup>

Total DNA of *S. griseus* was digested with each of *Sph* I, *Sst* I and *Bgl* II for ligation into the corresponding site within the tyrosinase gene of pIJ 702<sup>6)</sup> (supplied by Dr. C. THOMPSON).

Vector (4  $\mu$ g) and donor DNA (8  $\mu$ g) were mixed and digested with restriction endonuclease then ligated at 4°C overnight with T4 DNA

ligase.

Preparation of protoplast and transformation of *S. lividans* 1326 were as described by CHATER *et al.*<sup>5)</sup>

After plating for 16 hours R2YE plates were overlaid with R2YE containing thiostrepton. Clones resistant to thiostrepton were replicated to ISP No. 4 plates containing 0.1% yeast extract and 20 or 50  $\mu$ g/ml SM.

Preparation of cell-free extract and inactivation of SM were as described by NIMI *et al.*<sup>7)</sup> with the following modifications.

SM resistance clones were grown in YEME<sup>5)</sup> medium containing 5  $\mu$ g/ml SM. Ten grams of the mycelium was washed and suspended in 40 ml reaction buffer (125 mM Tris-malate and 12.5 mM MgSO<sub>4</sub>, pH 7.0). The mycelium was disrupted with an ultrasonic oscillator in an ice bath. After centrifugation at 16,000 rpm for 10 minutes, 600 mg ATP, 200 mg SM sulfate and 1 ml toluene were added to the supernatant. Reactions were carried out at 37°C for 16 hours.

SM resistance clones only appeared in transformants of *S. lividans* with recombinant plasmids inserted with *Bgl* II fragments of total DNA of *S. griseus*. Such clones failed to appear in transformants with *Sph* I and *Sst* I fragments. Accordingly, *Bgl* II fragments of *S. griseus* and pIJ 702 linearized with *Bgl* II were ligated and gave the recombinant plasmid, pST 141, after transformation into *S. lividans*. Recombinant plasmid pST 141 was digested with restriction endonucleases and analyzed by agarose gel electrophoresis. It was found that a 7.0 Kb *Bgl* II fragment of *S. griseus* encoded the SM resistance determinant. A restriction map of the fragment is shown in Fig. 1.

The recombinant plasmid pST 141 was able to re-transform *S. lividans* 1326 protoplasts to thiostrepton and SM resistance, and the resistance to thiostrepton and SM was cured simultaneously when transformants were incubated without selection at 37°C.

The resistance level of the transformants is

Fig. 1. Restriction map of SM resistance fragment.

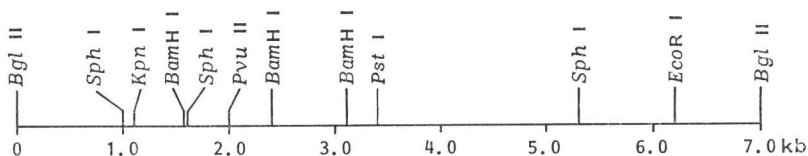


Table 1. MIC of streptomycin ( $\mu\text{g/ml}$ ) on *S. lividans* transformant and *S. griseus*.

Strain	Plasmid	MIC ( $\mu\text{g/ml}$ )
<i>S. lividans</i> 1326	None	3
3131	pIJ 702	12
4-1	pST 141	200
<i>S. griseus</i> ISP 5236	None	100

Agar dilution method.

shown in Table 1 as minimum inhibitory concentration (MIC) to SM. *S. lividans* 4-1 harboring pST 141 and *S. griseus* ISP 5236 (donor strain) were resistant to SM, while *S. lividans* 1326 (recipient strain) and 3131 harboring pIJ 702 were sensitive. The susceptibility to SM in SM resistant clones of *S. lividans* was increased 10 fold or more over that of SM sensitive hosts.

Inactivated SM was isolated from a cell-free reaction mixture, purified by column chromatography on CM-Sephadex C-25 and LH-20, then subjected to  $^{13}\text{C}$  NMR and SI-MS analysis. It gave positive color reaction with the ammonium molybdate-perchloric acid reagent. SI-MS,  $m/z$  662 ( $\text{M}+1$ )<sup>+</sup>, showed the presence of phosphorous in this molecule. From  $^{13}\text{C}$  NMR spectrum the signal of 6-C<sup>(5)</sup> was shifted to lower field by 3.6 ppm and split by coupling with the phosphorous group. Therefore, it was identified as SM 6-O-phosphate<sup>9)</sup>.

Since the fragment encoding SM resistance gene has no *Sst* I site, we presume that *Sst* I fragments encoding SM resistance determinant of *S. griseus* are too long to be stable in the host when inserted into pIJ 702. Similarly, the SM resistance transformants harboring pST 141 (12.5 Kb) frequently lost their plasmids. Therefore, subcloning of the *Bgl* II fragment containing the SM O-phosphotransferase gene will be necessary to stabilize recombinant plasmid in the host. The *Bgl* II fragment has three *Sph* I sites but since no resistance transformant appeared when *Sph* I fragments from *S. griseus* were cloned, this suggests that at least one of

these sites may be within the resistance gene.

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#### References

- 1) THOMPSON, C.J.; J.M. WARD & D.A. HOPWOOD: Cloning of antibiotic resistance and nutritional genes in *Streptomyces*. J. Bacteriol. 151: 668~677, 1982
- 2) BIBB, M. J.; J. L. SCHOTTEL & S. N. COHEN: A DNA cloning system for interspecies gene transfer in antibiotic-producing *Streptomyces*. Nature 284: 526~531, 1980
- 3) MURAKAMI, T.; C. NOJIRI, H. TOYAMA, E. HAYASHI, K. KATUMATA, H. ANZAI, Y. MATSUHASHI, Y. YAMADA & K. NAGAOKA: Cloning of antibiotic-resistance genes in *Streptomyces*. J. Antibiotics 36: 1305~1311, 1983
- 4) NAKANO, M.; H. MASHIKO & H. OGAWARA: Cloning of the kanamycin resistance gene from a kanamycin-producing *Streptomyces* species. J. Bacteriol. 157: 79~83, 1984
- 5) CHATER, K. F.; D. A. HOPWOOD, T. KIESER & C. J. THOMPSON: Gene cloning in *Streptomyces*. Curr. Topics Microbiol. Immunol. 96: 69~95, 1981
- 6) KATZ, E.; C. J. THOMPSON & D. A. HOPWOOD: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129: 2703~2714, 1983
- 7) NIMI, O.; G. ITO, S. SUEDA & R. NOMI: Phosphorylation of streptomycin at C<sub>6</sub>-OH of streptidine moiety by an intracellular enzyme of *Streptomyces griseus*. Agric. Biol. Chem. 35: 848~855, 1971
- 8) MUNRO, M. H. G.; R. M. STROSHANE & K. L. RINEHART, Jr.: Location of guanidino and ureido groups in bluensomycin from  $^{13}\text{C}$  NMR spectra of streptomycin and related compounds. J. Antibiotics 35: 1331~1337, 1982
- 9) SUGIYAMA, M.; H. MOCHIZUKI, O. NIMI & R. NOMI: Mechanism of protection of protein synthesis against streptomycin inhibition in a producing strain. J. Antibiotics 34: 1183~1188, 1981