Molecular cloning and expression in *Streptomyces lividans* of a streptomycin 6-phosphotransferase gene from a streptomycin-producing microorganism

Hidenori Shinkawa, Masanori Sugiyama*, Osamu Nimi and Ryosaku Nomi

Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Saijo-cho, Higashi-Hiroshima 724, Japan

Received 7 November 1984; revised version received 2 January 1985

The gene encoding streptomycin 6-kinase involved in the self-resistance of the streptomycin-producing *Streptomyces griseus* HUT 6037 was cloned in the plasmid vector pIJ703. The resulting plasmid, pSP6, contained 2.5 kb inserts of *S. griseus* DNA. When streptomycin-susceptible *S. lividans* 1326 was retransformed with pSP6, all transformants produced streptomycin 6-kinase. Addition of streptomycin to the culture medium of *S. lividans* carrying pSP6 plasmid brought about a remarkable increase in streptomycin 6-kinase activity in the cell extracts. It is suggested from the results that the production of streptomycin 6-kinase in streptomycin producer was induced by streptomycin accumulated during cultivation.

Self-resistance *Streptomycin* *Streptomycin 6-kinase*

1. INTRODUCTION

Streptomycin-producing microorganisms are inherently resistant to their own antibiotic product, at least during the production phase of growth. We demonstrated previously that while the ribosomes of streptomycin-producing *Streptomyces griseus* bound streptomycin [1,2], the bound antibiotic was released when phosphorylated with ATP:streptomycin 6-phosphotransferase (streptomycin 6-kinase) [3]. In addition, streptomycin 6-phosphate did not inhibit protein synthesis of the streptomycin producer [4]. The streptomycin 6-kinase was purified and physico-chemically characterized [5]. From these results, it was concluded that streptomycin 6-kinase was the major contributor to self-resistance in the streptomycin producer.

On the other hand, since production of streptomycin 6-kinase increases with formation of streptomycin, it is of interest to investigate whether the enzyme is induced by streptomycin accumulated during cultivation of the producing organism.

Here, we report the cloning and expression of a gene which determines a streptomycin-phosphorylating enzyme in the streptomycin-producing *S. griseus*. In addition, we discuss the possibility that the gene coding for the enzyme could be induced by streptomycin.

2. MATERIALS AND METHODS

2.1. *Microorganisms, chemicals and media*

The streptomycin-producing *S. griseus* HUT 6037 was used as a DNA donor strain. The strain was resistant to streptomycin up to 200 μg/ml on Fructose-Bennett-agar (FB) medium [6]. The recipient organism used as a host for recombinant plasmid was *S. lividans* 1326. The strain was sensitive to 5 μg/ml thiopeptin (one of the thiostrepton group antibiotics) and 5 μg/ml streptomycin. Since thiostrepton was not available, thiopeptin supplied by Fujisawa Pharmaceutical Co. was used

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

for the selection of thiostrepton-resistant transformants.

2.2. *DNA preparation*

Total DNA was isolated from mycelium of *S. griseus* using the procedure of Marmur [7]. Detection and isolation of plasmid DNA were carried out according to the alkaline denaturation method of Omura et al. [8].

2.3. *Procedure of shotgun cloning*

Total DNA from *S. griseus* HUT 6037 was partially digested with restriction endonuclease *Bam*HI, and ligated to *Bgl*II-cleaved pIJ703 using T4-DNA ligase [9]. The pIJ703 carries the genes specifying thiostrepton resistance and tyrosinase enzyme responsible for melanin synthesis [9]. The *Bgl*II site within the tyrosinase gene was convenient since it enabled the use of insertional inactivation of melanin production for clone recognition.

2.4. *Preparation and transformation of protoplasts*

These were principally carried out as described by Thompson et al. [10].

2.5. *Assay of streptomycin 6-kinase activity*

Streptomycin 6-kinase activity was determined by two methods as in [5]. Method A involved determination of the amount of streptomycin 6-[^{32}P]phosphate produced by the enzyme from streptomycin and [γ-^{32}P]ATP. Method B involved determination of the decreased amount of streptomycin after phosphorylation of the drug by the enzyme.

3. RESULTS AND DISCUSSION

To clone the gene coding for streptomycin 6-kinase, total DNA from *S. griseus* HUT 6037 was partially digested with *Bam*HI and ligated to *Bgl*II-cleaved pIJ703. After introduction of the ligated DNA into protoplasts of *S. lividans* 1326, regeneration of the protoplasts was carried out on

Table 1

Characteristics of *Streptomyces* strains described in the text

Strain	Plasmid	Insertion to pIJ703 (kb)	Specific activity of streptomycin 6-kinase[a]
S. griseus HUT 6037	–	–	0.95
S. lividans 1326	–	–	0.00
S. lividans 3132	pIJ703	–	0.00
S. lividans SMR-1	pSP1	5.4	0.63
S. lividans SMR-2	pSP2	5.8	0.42
S. lividans SMR-3	pSP3	4.4	0.31
S. lividans SMR-4	pSP4	5.4	0.47
S. lividans SMR-5	pSP5	5.4	0.31
S. lividans SMR-6	pSP6	2.5	0.76

[a] Specific activity of streptomycin 6-kinase is expressed as μmol streptomycin 6-phosphate formed in 1 h by 1 mg protein in the S-30 fraction

For the determination of streptomycin 6-kinase, *S. lividans* 3132 was grown in YEME medium [11] and *S. lividans* transformants (strains SMR-1 to SMR-6) in the same medium containing thiopeptin (50 μg/ml) and streptomycin (5 μg/ml), and they were harvested at the middle of the exponential phase of growth. *S. griseus* HUT 6037 grown in GMP medium (1% glucose, 0.5% NaCl, 0.4% peptone, 0.2% yeast extract, 0.2% meat extract and 0.025% $MgSO_4 \cdot 7H_2O$, pH 7.0) was harvested at a growth phase producing 5 μg/ml streptomycin. Streptomycin 6-kinase activity was determined by using supernatant fluids (S-30 fractions) obtained by centrifuging the extracts from harvested cells at 30000 $\times$ *g* for 30 min. Streptomycin 6-kinase activities were determined by method A

R2YE medium [10]. Among about 15000 colonies which were phenotypically thiopeptin resistant, 16 were resistant to both thiopeptin (50 μg/ml) and streptomycin (20 μg/ml), and produced no melanin pigment. They were then examined for the production of streptomycin 6-kinase activity. As shown in table 1 and fig.1, the cell extracts (S-30 fraction) from the 6 transformants named *S. lividans* SMR-1 to SMR-6 contained significant amounts of the enzyme, though the activity was somewhat lower than that from *S. griseus* HUT 6037. The 10 other transformants did not contain streptomycin 6-kinase activity and the growth was significantly derepressed on FB agar medium containing thiopeptin and streptomycin during successive transfer of the culture. Therefore, these clones were not further analyzed for resistance mechanisms to streptomycin. The plasmids were isolated from the 6 streptomycin 6-kinase-producing transformants and subjected to agarose gel electrophoresis. They migrated more slowly than the pIJ703 vector plasmid. The plasmids of *S. lividans* SMR-1 to SMR-6 were named pSP1 to pSP6, respectively. Single and double digestions of these plasmids by different restriction endonucleases revealed that plasmids pSP1, 4 and 5 contained the same 5.4 kb inserts from *S. griseus* DNA, whereas pSP2, 3 and 6 had a 5.8, 4.4 and 2.5 kb insertion, respectively (table 1). We assumed that these inserted DNAs carried an identical copy of a streptomycin 6-kinase gene. A partial restriction map of pSP6, which has the smallest insertion of DNA among those plasmids, is illustrated in fig.2. The streptomycin 6-kinase fragment (2.5 kb) possessed one cleavage site each for *Bcl*I and *Sac*I, and 4 sites for *Sma*I. There were no recognition sites for *Bam*HI, *Bgl*II, *Pst*I and *Xho*I in the streptomycin 6-kinase gene. Furthermore retransformation of *S. lividans* 1326 with pSP6 yielded transformants which were resistant to both thiopeptin and streptomycin.

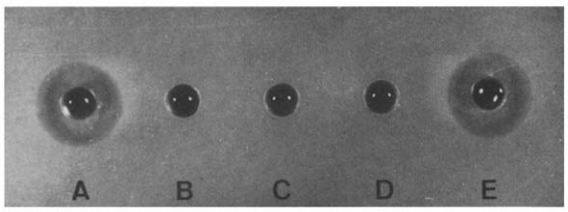

Fig.1. Inactivation of streptomycin by the S-30 fraction of different *Streptomyces* strains. The assay plate for antibiotic activity consisted of meat extract (0.3%)-peptone (0.5%)-agar and *Bacillus subtilis* IFO 3134 as a test organism. The reaction mixture for the determination of streptomycin 6-kinase activity by method B consisted of 0.2 ml of 5 mM streptomycin, 0.2 ml of 15 mM ATP, 0.1 ml of 100 mM $MgSO_4$, 0.1 ml of 1 M Tris-maleate (pH 7.0) and 0.4 ml of S-30 fraction. The mixture was incubated at 30°C for 1 h, then the reaction was stopped by heating at 100°C for 5 min. After a 100-fold dilution of the mixture with 0.1 M phosphate buffer (pH 7.8), the antibiotic activity was assayed. (A) Control; S-30 fraction in the reaction mixture was replaced by buffer consisting of 50 mM Tris-HCl (pH 8.0), 10 mM $MgSO_4$, 0.5 mM dithiothreitol and 0.4 mM phenylmethylsulfonyl fluoride. (B) S-30 fraction from *S. griseus* HUT 6037. (C) S-30 fraction from SMR-1 strain. (D) S-30 fraction from strain SMR-6. (E) S-30 fraction from *S. lividans* 1326.

Streptomycin-producing organisms have been reported to be significantly sensitive to their own antibiotic at the early stage of growth. However resistance to streptomycin develops during growth. Fig.3a shows the time course of growth and of the production of streptomycin and streptomycin 6-kinase. The production of the enzyme increased with the formation of streptomycin, suggesting that the enzyme was induced by streptomycin accumulated in the broth. This finding encouraged us to investigate the regulation mechanism for the

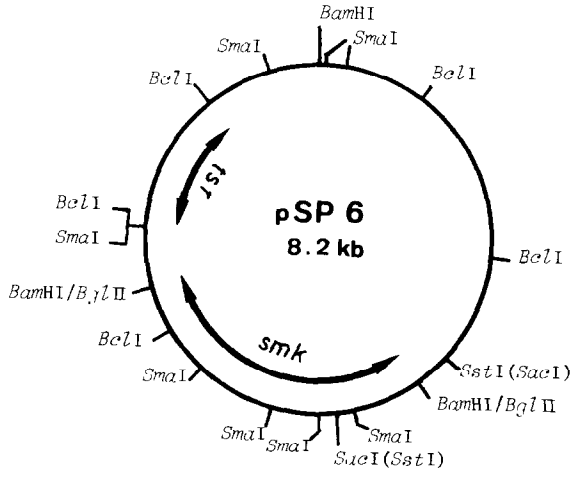

Fig.2. Restriction endonuclease map of pSP6 plasmid. *tsr*, thiostrepton resistance gene; *smk*, streptomycin 6-kinase gene.

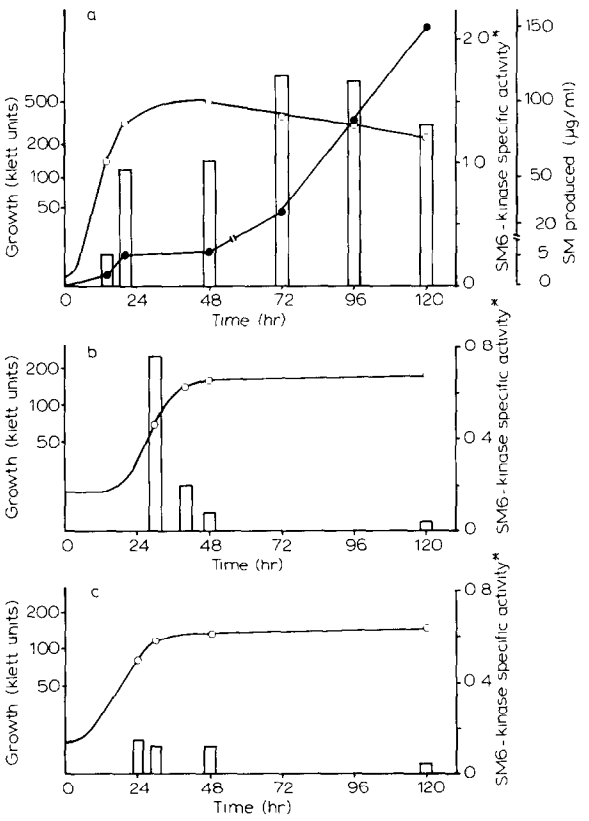

Fig.3. The time course of streptomycin 6-kinase production by *S. griseus* HUT 6037 and *S. lividans* carrying pSP6 plasmid. *S. griseus* HUT 6037 (a) was grown in GMP medium. *S. lividans* carrying pSP6 was grown in YEME medium [11] containing thiopeptin (50 μg/ml) and streptomycin (5 μg/ml) (b), or in the same medium containing thiopeptin only (c). Addition of thiopeptin was necessary for stable replication of the plasmid. The YEME medium was preferable to GMP medium for the growth of *S. lividans*. SM, streptomycin. Streptomycin 6-kinase activity was determined by method A. * The specific activity is expressed as in table 1. (○—○) Growth, (●—●) streptomycin produced in the broth, (vertical bars) specific activity of streptomycin 6-kinase.

production of streptomycin 6-kinase using the above transformant. *S. lividans* SMR-6 was cultivated in YEME medium [11] containing both thiopeptin (50 μg/ml) and streptomycin (5 μg/ml) or the same medium which contained thiopeptin only. Thiopeptin was used to stabilize replication of the plasmid. The time courses of growth and production of streptomycin 6-kinase are shown in fig.3b and c. Addition of streptomycin to the culture of *S. lividans* SMR-6 gave rise to a remarkable increase in specific activity of streptomycin 6-kinase. It was presumed from these results that the expression of the streptomycin 6-kinase gene was induced by streptomycin added. However, it is strange that the enzyme activity decreased to the same level as in the sample without addition of streptomycin after an initial increase (fig.3b and c). Since streptomycin added to the medium is inactivated by the product of the introduced gene (streptomycin 6-kinase), it is conceivable that subsequent increase in enzyme production would stop soon after induced production. However, it is not clear why the activity of the pre-existing enzyme decreased. The life-span of the enzyme might be short.

Using a coupled transcription-translation system consisting of the S-30 fraction from *S. lividans* 1326 and pSP6 plasmid as a template DNA, an experiment is now in progress to determine whether streptomycin 6-kinase is produced in the reaction mixture and whether production of the enzyme increases by addition of streptomycin to the reaction mixture.

ACKNOWLEDGEMENTS

We wish to thank Dr E. Katz, Georgetown University Schools of Medicine and Dentistry, USA, for the gift of *S. lividans* carrying pIJ703. We are grateful to Fujisawa Pharmaceutical Co. for a kind gift of thiopeptin. This work was supported in part by a grant from the Ministry of Education, Japan (59750755).

REFERENCES

[1] Sugiyama, M., Kobayashi, H., Nimi, O. and Nomi, R. (1980) FEBS Lett. 110, 250–252.
[2] Sugiyama, M., Mochizuki, H., Nimi, O. and Nomi, R. (1981) Biotechnol. Lett. 3, 357–362.
[3] Sugiyama, M., Mochizuki, H., Nimi, O. and Nomi, R. (1981) J. Antibiot. 34, 1183–1188.
[4] Sugiyama, M., Mochizuki, H., Nimi, O. and Nomi, R. (1982) FEBS Lett. 139, 331–333.
[5] Sugiyama, M., Sakamoto, M., Mochizuki, H., Nimi, O. and Nomi, R. (1983) J. Gen. Microbiol. 129, 1683–1687.

[6] Sugiyama, M., Katoh, T., Mochizuki, H., Nimi, O. and Nomi, R. (1983) J. Ferment. Technol. 61, 347–351.
[7] Marmur, J. (1961) J. Mol. Biol. 3, 208–218.
[8] Omura, S., Ikeda, H. and Tanaka, H. (1981) J. Antibiot. 34, 478–482.
[9] Katz, E., Thompson, C.J. and Hopwood, D.A. (1983) J. Gen. Microbiol. 129, 2703–2714.
[10] Thompson, C.J., Ward, J.M. and Hopwood, D.A. (1982) J. Bacteriol. 151, 668–677.
[11] Bibb, M.J., Freeman, R.F. and Hopwood, D.A. (1977) Mol. Gen. Genet. 154, 155–166.