

Introduction of Kanamycin Resistant Gene to Pock-forming Plasmid pSA1.1 of *Streptomyces azureus* and Identification of spoIIIE-like Gene in the Recombinant Plasmid

Doi, Katsumi

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

Tomura, Takafumi

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

Kishino, Hiroko

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

Hara, Toshio

Microbial Genetics Division, Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

他

<https://doi.org/10.5109/24051>

出版情報：九州大学大学院農学研究院紀要. 38 (3/4), pp.193-205, 1994-03. Kyushu University
バージョン：
権利関係：



Introduction of Kanamycin Resistant Gene to Pock-forming Plasmid pSA1.1 of *Streptomyces azureus* and Identification of *spoIIIE*-like Gene in the Recombinant Plasmid

Katsumi Doi, Takafumi Tomura, Hiroko Kishino,
Toshio Hara, Satoru Kuhara,*¹ Kohzo Kanda*² and Seiya Ogata⁺

Microbial Genetics Division, Institute of Genetic Resources and *¹Laboratory of Molecular Gene Technics, Graduate School of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan

(Received November 24, 1993)

A plasmid, pSA1.1, of *Streptomyces azureus* elicited conjugative pocks and inhibited the sporulation of its host mycelia. The analysis of plasmid pSA1.1 was hampered by the lack of selective marker. Therefore, the introduction of a kanamycin resistance gene (*kanr*) derived from *Streptomyces kanamyceticus* to pSA1.1 was tried. The obtained recombinant still preserved the sporulation-inhibitory and pock-forming abilities and was able to confer kanamycin resistance on the host strain. The sporulation-inhibitory gene was studied by the use of one of these recombinants.

The deletion analysis and sequencing of the derivative plasmid found an open reading frame (ORF909b) that was involved in sporulation-inhibitory function of pSA1.1 and in pock formation. The nucleotide sequences of ORF909b were compared with those of the genes registered in GenBank, but no similarity was found. However, the predicted amino acid sequence of ORF909b showed a significantly high similarity with that of the *spoIIIE* gene of *Bacillus subtilis*. This detected gene might be a new sporulation-regulatory gene in streptomycetes. The function of the detected gene on the sporulation-inhibition was also discussed.

INTRODUCTION

When a *Streptomyces* spore or mycelial fragment carrying a conjugative plasmid grows on a solid medium in contact with a plasmid-free strain, transfer of the plasmid into the plasmid-free mycelium occurs. The growth and/or sporulation of the newly infected mycelium is delayed, and a circular area of retarded development around the colonies or lawns developed from the plasmid-harboring spore or mycelium is seen. This phenomenon is called pock formation and such a plasmid is called a pock-forming plasmid. Functional and sequence analysis of the pock-forming plasmids pIJ101, pSAM2 and pSN22 (Kieser *et al.*, 1982; Kendall *et al.*, 1987, 1988; Stein *et al.*, 1989; Smokvina *et al.*, 1991; Kataoka *et al.*, 1991a, b) have been done.

Plasmid pSA1.1-harboring mycelia (or spores) of *Streptomyces azureus* elicited conjugative pocks toward the plasmid-free mycelia at a frequency of 100% (Miyoshi *et al.*, 1986). The pSA1.1 also inhibited the sporulation of its host mycelia (Miyoshi *et al.*, 1986). This sporulation-inhibitory function of pSA1.1 might be involved in the pock formation. The functional analysis of the pSA1.1 would provide a new aspect of studies of pock formation and feature or mechanisms of *Streptomyces* sporulation.

However, the determination and analysis of the region concerned with the

*²Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Saga 840.

+ To whom all correspondence should be addressed.

sporulation- inhibitory function of plasmid pSA1.1 was difficult, because it has no efficient selective marker except its own pock formation. In many gene manipulations of *Stqbtomyces*, Thiostrepton resistance gene (*tsr*) (Thompson *et al.*, 1982) have been used as useful selective marker. But, as *S. azureus* is a producer of thiostrepton, *tsr* gene should be located on the chromosome of this strain. Therefore, another selective marker was need to introduce into the pSA1.1. In this paper, we describe the introduction of new selective marker to the pSA1.1, and then the analysis of its sporulation-inhibitory gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Stqbtomyces azureus* PKIOOC, a plasmid - free strain of *S. azureus* ATCC 14921, was used as a host or recipient strain in the pock-forming assay (Miyoshi *et al.*, 1986). Strains 1326 and TK21 of *Stqbtomyces lividans* were used as other *Streptomyces* hosts and control strains of kanamycin resistance assay. *Escherichia coli* JM109 was used as the *E. coli* host throughout this study (Yanisch-Perron *et al.*, 1985). The *Stqbtomyces* plasmids pSA1.1 (Miyoshi *et al.*, 1986), a high copy number derivative of pSA1 of the wild type strain, and the *Stqbtomyces* vector pMCP5 (Nakano *et al.*, 1984), pANT3- 1 (Hotta *et al.*, 1988) and the *E. coli* vector pUC19 (Yanisch-Perron *et al.*, 1985) have been described. All other plasmids were constructed during this study and there were described in the text.

Media and culture conditions. *S. azureus* was grown at 28°C on Rye flakes agar (Ogata *et al.*, 1981) for sporulation and pock-forming assay, in MG broth (Ogata *et al.*, 1983) for extraction of plasmids, in YEMES broth (YEME broth plus 34% sucrose) (Chater *et al.*, 1982) for preparation of protoplasts, on R2YE agar (Chater *et al.*, 1982) for protoplast regeneration. Glycine was added to 0.4% (w/v) to the liquid media MG broth and YEMES broth. *S. lividans* was grown at same condition. *E. coli* JM109 was grown at 37°C in LB broth (Maniatis *et al.*, 1982) or on LB agar. *Streptomyces* plasmids except pSA1.1 were selected by using kanamycin (25 µg/ml). The derivatives of pUC19 were selected in *E. coli* by using ampicillin (50 µg/ml).

Assay of antibiotic resistance and detection of survival rate. Detection of antibiotic resistance of strain PKIOOC of *Stqbtomyces azureus* was performed by modifying the gradient plate method described by Szybalski *et al.* (1952). On Bennett agar gradient plate containing antibiotics, spore suspension of strain PKIOOC were streaked. Antibiotics used in this experiment were as follows; penicillin G and ampicillin as penicillins, kanamycin, streptomycin and gentamicin as aminoglycoside, terramycin as tetracyclines, erythromycin as macrolide antibiotics, naramycin B, chloramphenicol, rifamycin as glutarimide antibiotics, thiostrepton (main products of *S. azureus*) as control. To detect survival rate of strain PKIOOC against each antibiotics, spore suspension (5×10^5 cfu/plate) was spread on Rye flake agar plate containing antibiotics, and after incubation, the number of colonies grown was counted.

The same experiments were also done on the transformants of strain PKIOOC and *S. lividans* and its derivatives.

DNA isolation, manipulations and transformation. Isolation of plasmid DNA from *S. azureus* and from *E. coli* was performed according to the description by Birnboim and

Doly (1979) and Kieser (1984), respectively. *S. azureus* was transformed by the method described previously (Ogata *et al.*, 1984), and *E. coli* was transformed by the method of Maniatis *et al* (1982).

Detection of sporulation. Coloring (bluish green) of colonies or lawns of the plate culture was estimated as the degree of sporulation. Further the number of spores was estimated by microscopic counts of 10 random haemocytometer fields as described previously (Ogata *et al.*, 1992). For this detection, Rye flakes agar plate was used; Rye flakes agar medium was more suitable than the other medium for the formation of aerial mycelium and sporulating hyphae of *S. azureus*.

DNA sequencing. DNA fragments were subcloned into plasmid pUC19, and DNA sequencing was done by the dideoxy chain termination method (Sanger *et al.*, 1977) with Sequenase version 2.0 DNA sequencing kit using 7-deaza-dGTP (United States Biochemical Corporation, Ohio, U.S.A.). The sequence data was analyzed with the DNASIS, GENETYX, and BLAST software programs.

RESULTS

Resistance of *S. azureus* to antibiotics

As analysis of plasmid pSA1.1 was hampered by the lack of selective marker except its own pock formation, we tried to introduce selective marker such as antibiotic resistance gene into pSA1.1. At first, the resistibility of strains PK100C and PK100C(pSA1.1) of *S. azureus* to some antibiotics was examined. As shown in Fig. 1, no resistibility of both strains toward kanamycin, streptomycin, erythromycin, terramycin, rifamycin were detected. It was considered that the resistance genes for

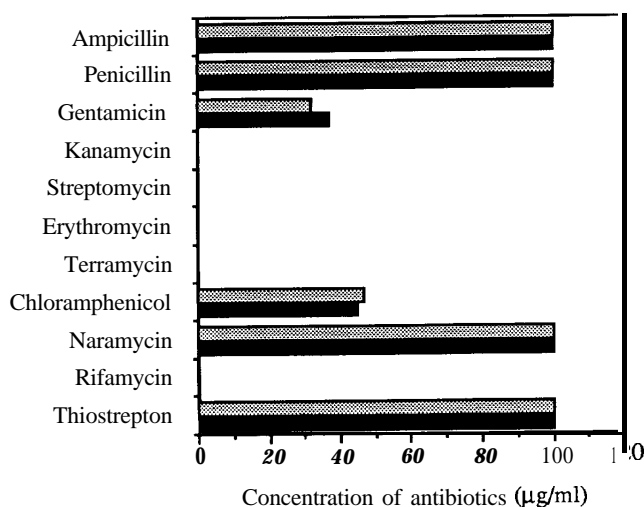


Fig. 1. Resistance of the derivatives of *Streptomyces azureus* ATCC14921 to various antibiotics: ▨, resistibility of strain PK100C; ■, resistibility of strain PK100C(pSA1.1).

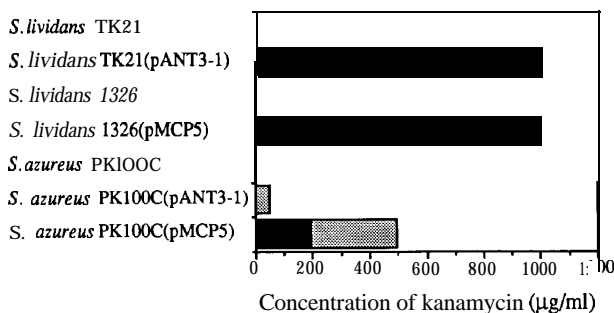


Fig. 2. Kanamycin-resistibility of transformants with *Streptomyces* plasmid vectors, ■, survival rate of 70% or more; ▨, survival rate of 20% to 70%.

these antibiotics were used as selective marker of plasmid pSA1.1 and its host, PK100C.

Resistance of transformants to Kanamycin

Two different resistance genes for kanamycin (*kmr*) had been cloned into *Streptomyces* vector pIJ702: One, pMCP5, was isolated from *Streptomyces kanamyceticus* ISP5500, and the other, pANT3-1, from *Streptomyces griseus* SS-1198PR. To confirm whether these *kmr* genes could express in *S. azureus*, strain PK100C were transformed with pMCP5 or pANT3-1. The transformants with kanamycin resistibility were obtained by use of each plasmids, respectively, as shown in Fig. 2. Both plasmids showed the same significant effect on the expression of kanamycin resistance in *S. lividans*, but did the lesser and different effect on that of *S. azureus*. The survival rate of PK100C (pMCP5) was higher than that of PK100C (pANT3-1), and lower than that of *S. lividans* 1326 (pMCP5) used as control. We will touch upon this different response and lesser effect in *S. azureus* than in *S. lividans* in Discussion. However, *kmr* gene from plasmid vector pMCP5 seemed to be able to apply as useful selective marker in plasmid pSA1.1 and its host, *S. azureus*.

Addition of *kmr* gene to plasmid pSA1.1

To analyze the function of plasmid pSA1.1, introduction of the *kmr* gene into pSA1.1 was tried. When the small *Barn* HI fragment of pSA1.1, about 40 -bp (Fig. 3), was deleted, no significant change occurred in its features. Therefore, the *Bcl* I fragments of plasmid pMCP5, one of which contained the *kmr* gene, was cloned into the *Barn* HI site of plasmid pSA1.1 in strain PK100C. The kanamycin - resistant transformant obtained carried the recombinant plasmid pSAK1, as shown in Fig. 3. The pSAK1 consisted of pSA1.1 and *Bcl* I fragments of 1.1- kb and 4.0-kb (containing *kmr* gene).

The recombinant plasmid pSAK1 was able to confer kanamycin resistance on the host strain PK100C up to 250 µ g/ml, although the pSA1.1 -harboring PK100C was inhibited at less than 5 µ g/ml. And, the kanamycin resistibility of pMCP5 -harboring PK100C and pSAK1 -harboring PK100C were almost the same (Fig. 3 and Fig. 4). The pSAK1 still preserved the sporulation -inhibitory and pock-forming abilities, *i.e.*, more than 5×10^8 spores/slant and less than 5×10^4 spores/slant were produced by strain PK100C and by its pSAK1, as reported previously (Ogata *et al.*, 1992). The following

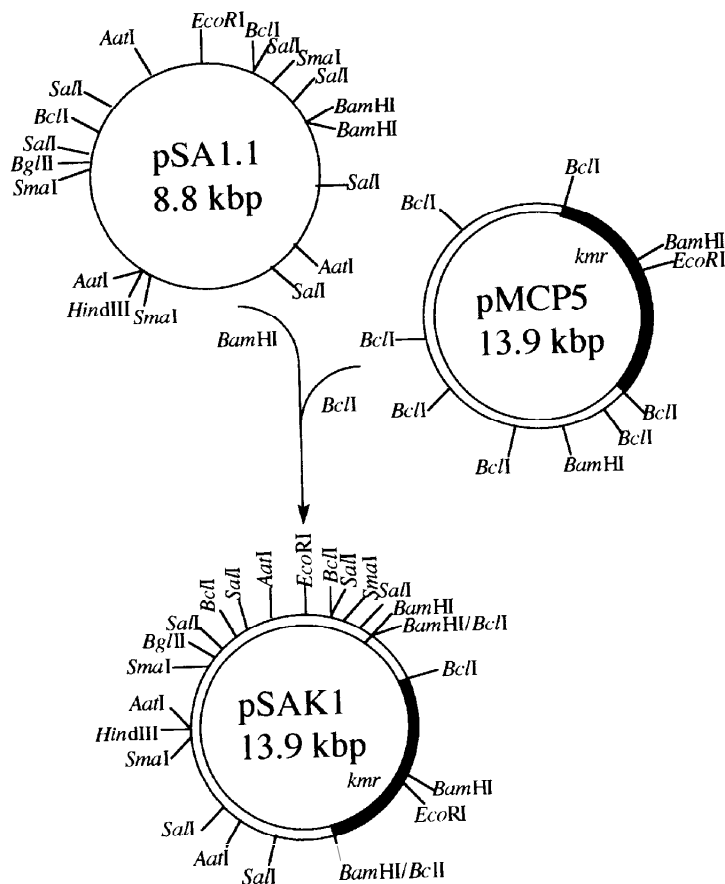


Fig. 3. Construction of plasmid pSAK1 with kanamycin resistant gene (*kmr*)

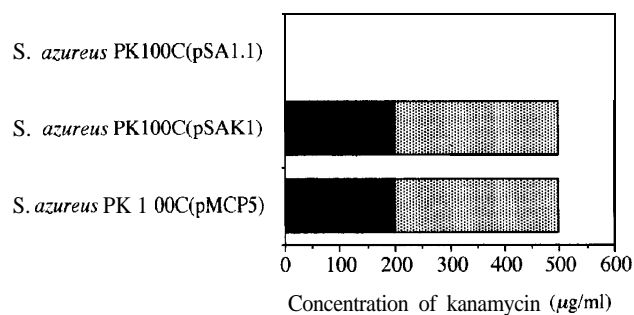


Fig. 4. Kanamycin resistibility of transformants with plasmid pSA1.1 and its derivatives, pSAK1, and the plasmid vector pMCP5. ■ , survival rate of 70% or more; ▨, survival rate of 20% to 70%.

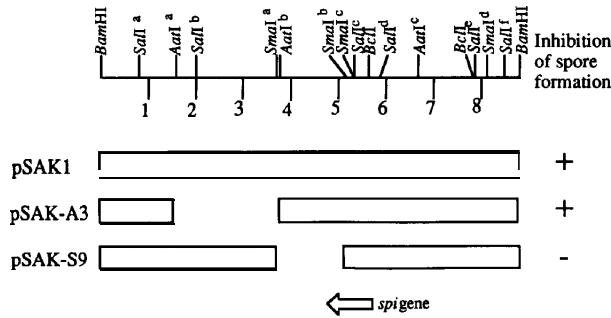


Fig. 5. Deletion analysis of pSAK1. The restriction map of pSA1.1 is shown on the top. The derivatives of pSAK1 are shown as pSAK-A3 and pSAK-S9. They were made by deletion with the appropriate restriction enzymes. +, inhibition of spore formation; -, no inhibition of spore formation. The location and orientation of *spi* gene is showed by a horizontal arrow.

experiments were done with pSAK1.

Location of sporulation-inhibitory gene on pSA1.1

To analyze the loci concerned with the inhibition of sporulation, the deletion mutants pSAK-A3 and pSAK-S9 were derived from the recombinant plasmid pSAK1 (Fig. 5). The pSAK-A3 and pSAK-S9 lacked the 2.1-kb *AatI*^I-*AatI*^I fragment and 1.6kb *SmaI*^I-*SmaI*^I fragment, respectively. The strain PK100C was transformed with each deletion plasmid. To examine their ability to inhibit the sporulation of its host mycelia, the transformants were spread on Rye flakes agar containing kanamycin (25 µg/ml). As shown in Fig. 5, pSAK-A3 inhibited the sporulation of its host mycelia,

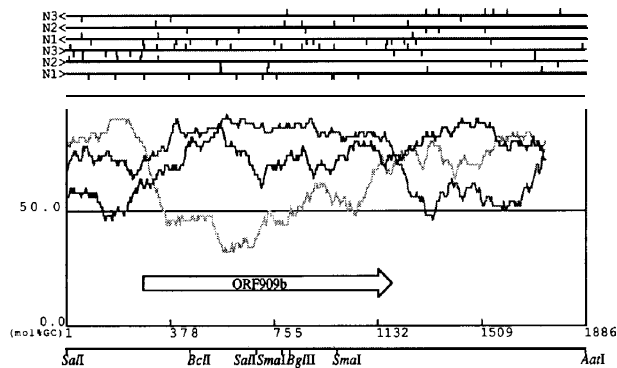


Fig. 6. Frame analysis of *AatI*^b-*SalI*^d fragment. The average of mol% G+C over a 50-codon window is plotted for the first (···), second (.....), and third (—) position in each triplet. The positions of start codon (⊥) and stop codon (⊥) for each possible reading frame (N1>, N2>, N3>, N1<, N2<, and N3<) are indicated at the top. The position and orientation of ORF909b are shown by a horizontal arrow. The ORF909b is in the N3 > frame.

Fig. 7. DNA sequence and predicted amino acid sequence of the *spi* gene.

10	20	30	40	50	60	70	80	90
GTCGACGATGGATGTGTGTGGGCTGACGGTGACCCGTCGGGCTGCGGGGCTTCATGACCCGCAAGCTGCGCCCGCGTCGAGGTTCAGCC								
100	110	120	130	140	150	160	170	180
GGTCCGCGCGAAGATCGCGCGGATACGCGGTACCTCGACAGGGCTGCGGGTGACACTGCGACTTCGCGCGGCGCTGAGACCGCGCTGACGT								
190	200	210	220	230	240	250	260	270
GATGGCCTCCTCGGAGCGGCTCCGGCACGCGTGGGGTGTCCACTCGGTGACCGTGGTCGAGACCAAGCCGGTTTCGTGGAACTCGGGAT								
280	290	300	310	320	330	340	350	360
GACCGGCTATGACGTGCTGCGCGGGTCGCGATCCGCGGCAAGGCCCGCCCGCATGGCAATGGCGTTCGGGTCGGTGCGGAGGACGGGAC								
Y T C C A G C G S A A R P A R Y A W R S G R C A R T G R								
370	380	390	400	410	420	430	440	450
GCCTTCGAGCGGGACTACGCAAAAGCTCCGATGGCACTCACCTCGGCGCGCAACCACTCGGGCAAGTCGATGTATCAGCGCAACCTGATCA								
L R A G L R K A P M A L T L G A N H S G K S M Y Q R N L I K								
460	470	480	490	500	510	520	530	540
AGGGGCTGGCGCAACTGCGGTTGGCCCTGGTCGGTATCGACTGCAAGCGGGTGTGGAACAGGCCGCGTTGCGGCCCGGCTGTGCGGCG								
G L A O L P V A L V G I O C K R G V E O A A F A P R L S A L								
550	560	570	580	590	600	610	620	630
TGGTCACCACTCCGATGATGCGGCTCGCTGCTCGGTGTGCTGGTCCGCGAGATGGAGGGCCGCTTCGAGCTGCTGAGCCGTACGSGTG								
V T T P D D A A S L L G V L V A E W E G R F D L L S R H G V								
640	650	660	670	680	690	700	710	720
TCTCGAACCTGTGGAGCTGCTGCCGAGGTGCGCGGCTGCCGGTGGTCTGCTGTCGACGAGGTGCGCGGAACCTGTTCTTGATCTCCT								
S D L W E L P A E V R P Y P V V V L V D E V A E L F L I S S								
730	740	750	760	770	780	790	800	810
CCAGAAGGATGAGGAGCGCCGGGAACGGATCGTCACCGGTTGATCCGACTCGCGCAGATGGCCCGGGCCATCGGTATCCATCTGGAGA								
K K D E E R R E R I V T A L I R L A Q M A R A I G I H L E I								
820	830	840	850	860	870	880	890	900
TCTCGGCGAGCGCTTTGGCTCCGACCTGGGCAAGGCGCCACCATGCTCCGTGCTCAGCTACCGGCGGTGTGGTGACCGCGTCAAGC								
C G O R F G S O L G K G A T M L R A O L T G R V V H R V N O								
910	920	930	940	950	960	970	980	990
ACAAGCAGACCGCGAGATGGGTTTGGCGGACGTGGCCCCGGACCGCTACCGGCGCAAGCGCTGATCCCATGAACCGCCCGGGAGCCG								
K Q T A E M G L A D V A P D A V P A A S L I P M N R P G T A								
1000	1010	1020	1030	1040	1050	1060	1070	1080
CGGTGCGCGACACCGCTTCGCGTGGCTGGTCSAAGATCCGCAACCCGGGAACCTCACGGACGAAGTGATAGCGCTCGCGGGAGTTCG								
V A A D P S G G W S K I R T P E T S R D E V V A V C R E F A								
1090	1100	1110	1120	1130	1140	1150	1160	1170
CCGACCTTATCCCGGACCTGCGGTTCCTCGAAACCTTCGCGCCCGCGGTCGCGCGCGCGAGGTACCTGCGGCGGTCGTCGATGGTCAAGC								
H L I P D L P F L E P F R P R V P A E V P A A G P S M V K P								
1180	1190	1200	1210	1220	1230	1240	1250	1260
CCCGCCGCTCACCGATTAACCCCGCACCGGCCACAGTCGGACGCGTCGCGCCACCTTACCCCGCCATCGCGCTGGCGGCGCGGACCT								
R P I T F *								

Fig. 8. Amino acid sequence homology of *spi* product with *spoIIIE* product. Identical residues, similar residues, and gaps are indicated by asterisk, dots, and dashes, respectively. The boxed residues conform to the consensus sequence for nucleotide binding proteins.

would be concerned with the conjugative pocks.

Nucleotide sequence of the 1.9-kb *Aat*I^b-*Sal*I^d fragment

The nucleotides of the 1.9-kb *Aat*I^b-*Sal*I^d fragment, which includes the *Aat*I^b-*Sma*I^b fragment, were sequenced by dideoxy chain termination method. Analysis of the nucleotide sequence with the DNASIS software program found several open reading frames (ORFs). Frame analysis of sequenced *Aat*I^b-*Sal*I^d fragment was done by a GENETYX software program, as shown in Fig. 6. The G+C contents of the ORFs at the first, second, and third positions were also analyzed. The 909 - bases ORF (ORF909b) among several ORFs had only a G+C content fully characteristic of *Streptomyces* reading frames. The G+C contents of ORF909b showed 72, 52, and 84% at first, second, and third codons, respectively. Therefore, it is identified as the *spi* gene, of which the location and orientation are also shown in Figs. 5 and 6.

The nucleotide sequence of *spi* gene is presented in Fig. 7. The streptomycetes promoter sequence could not be found in the nucleotide sequence of 5' upstream of *spi* gene. The nucleotide sequence of the *spi* gene was compared with those of the genes

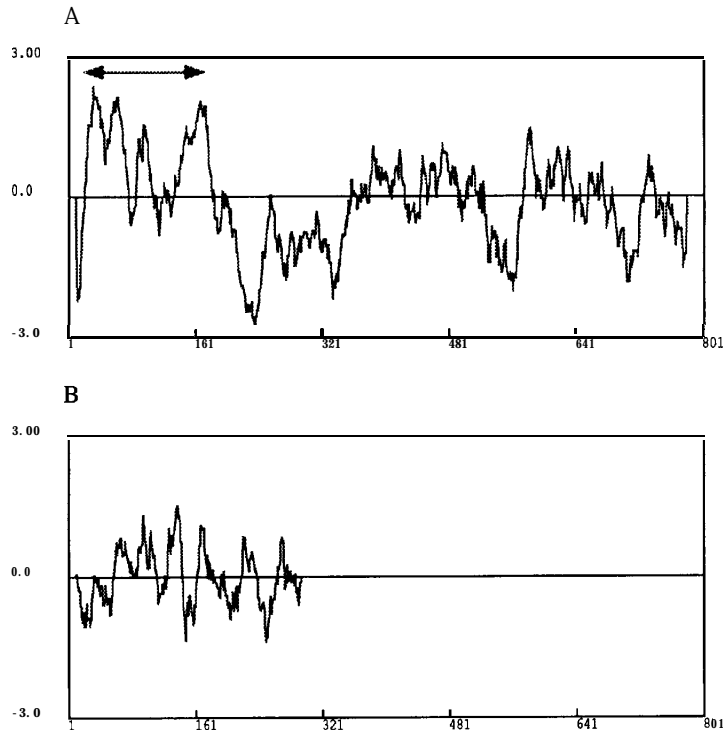


Fig. 9. Hydrophobicity / hydrophilicity plot of *spoIIIIE* protein and *spi* protein. The X-axis indicates the amino acid number from the start codon, and Y-axis indicates the relative hydrophobicity / hydrophilicity by method of Kyte and Doolittle. Consecutive hydrophobicity averages are plotted for a 20-residue window advancing from the NH₃ terminus to COOH terminus. A, *spoIIIIE* protein; B, *spi* protein; \longleftrightarrow , a hydrophobic region.

registered in GenBank by the BLAST software program, but no similarity was found.

Predicted amino acid sequence homology of *spi* gene product with *spoIIIE* gene product of *Bacillus subtilis*

The predicted amino acid sequence is also shown in Fig. 7. Computer analysis of the gene product indicated that the *spi* gene encoded a protein of *Mr* 32,513 containing 303 amino acid residues. The predicted amino acid sequence of the *spi* gene was compared with those of the genes and proteins registered in GenBank and PIR by the BLAST software program. The *spi* gene product and the *spoIIIE* gene product of *B. subtilis* had regions of highly conserved amino acid sequence (Fig. 8).

The highly conserved regions of amino acid sequence consisted of 261 amino acid residues. The location of the regions is also shown in Fig. 8. The region started at No.445 residue and ended at No.705 residue. The percent similarity and percent identity of the region were 65.1% and 27.1%, respectively. The region also had the consensus sequence for mononucleotide binding that had been described in the previous report on the *spoIIIE* gene (Foulger and Errington, 1989).

The hydrophobicity/hydrophilicity plot analysis (Kyte and Doolittle, 1982) revealed that the predicted *spoIIIE* gene product had the hydrophobic region which was recognized in adjacent to N-terminus (indicated as arrow in Fig. 9A). However, the similar region was not able to identify in the *spi* gene product (Fig. 9B).

DISCUSSION

A difficulty of genetic analysis of plasmid pSA1.1 of *Streptomyces azureus* was caused by the lack of useful selective markers. The resistibility of *S. azureus* toward various antibiotics was examined, and the kanamycin-resistibility was selected. And, it was clear that a kanamycin resistance gene (*kmr*) from plasmid vector pMCP5 was a efficient selective marker of plasmid pSA1.1 in *S. azureus*. We tested two kinds of the *kmr* genes from pMCP5, and pANT3-1, respectively. It was supposed that the different survival rates between PK100C(pMCP5) and PK100C(pANT3-1) would be due to their resistant mechanisms: the product of *kmr* of pMCP5 modifies 30S ribosomes (Nakano *et al.*, 1984), whereas the product of *kmr* of pANT3-1 directly acetylates the kanamycin (Hotta *et al.*, 1988). The lesser effect of *kmr* in *S. azureus* than in *S. lividans* would be due to the adaptation of *kmr* promoter in the host.

The sporulation-inhibitory gene (*spi*) of pock-forming plasmid pSA1.1 was studied by the addition of this *kmr* gene. Deletion of the recombinant plasmid led to the abundant spore formation by the host mycelia. Thus, it was possible to detect the location and nucleotide sequence of the *spi* gene. Comparison of the amino acid sequence showed that the *spi* gene and the *B. subtilis* *spoIIIE* gene were particularly closely related. The *spoIIIE* gene has a specific effect on *spoIIIG* expression: *spoIIIG* encodes a sigma factor that determines prespore -specific gene expression during sporulation of *B. subtilis* (Foulger and Errington, 1989). The *spoIIIE* gene is necessary for sporulation in *B. subtilis*. However, the *spi* gene inhibited sporulation in *S. azureus*.

An analogous situation had been reported for *Streptomyces* σ^{whiG} (controls the switch from continued extension of aerial hyphae to the development into chains of spores) and *B. subtilis* σ^D (regulates genes for motility and chemotaxis) (Chater, 1989;

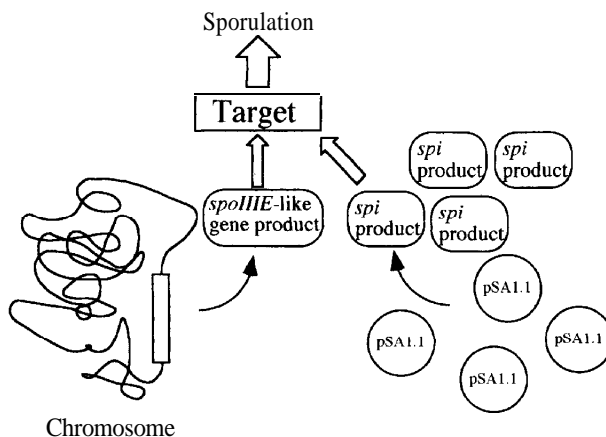


Fig.10. Schematic model for sporulation-inhibitory function of pSA1.1.

Chater et al., 1989). A DNA fragment carrying the σ^D -development protein inhibited sporulation when it was introduced into *S. coelicolor* with a high copy number plasmid. It is said that the inhibition of sporulation may be due to the reduction of an effective level of σ^{whiG} holoenzyme by binding to this foreign promoter. According to the above description and the similarity in the amino acid sequence between the *spi* gene and *spoIIIE* gene products, we supposed that the *spi* gene product might become a competitive inhibitor to the *spoIIIE*-like gene product which would be necessary for sporulation in *S. azureus* (Fig. 10). We were looked for the *spoIIIE*- like gene in the genomic DNA of *S. azureus* with the *spi* gene as a probe, and detected it (data are not shown, but we will report it soon).

The *spi* gene was considered to be involved in the pock formation, because the *spi* gene-lacking plasmid, pSAK-S9, did not form conjugative pocks. In the conjugative pocks, the *spi* gene should inhibit the sporulation of the recipient mycelia and form a circular are of retarded development around the door mycelia. No *spi*-like gene have been found in the pock-forming plasmids and genomic DNA of other *Streptomyces* species in the BLAST software analysis. We are now attempting to analyze the detailed functions of the *spi* gene and its adjacent genes in pSA1.1: A ORF in N1>frame is already found downstream from the *spi* gene (see Fig. 6). In the 1.9 - kb *Aat* I^b -*Sal* I^d fragment sequenced, we could not detect a promoter sequence of the *spi* gene. We are searching for it beyond the sequenced fragment.

ACKNOWLEDGEMENT

We are deeply grateful to Professor H. Ogawara (Meiji College of Pharmacy, Tokyo, Japan) and Doctor K. Hotta (National Institute of Health, Tokyo, Japan) for kindly supplying the *Streptomyces* plasmid pMCP5 and pANT3-1.

REFERENCES

- Birnboim, H. C. and J. Doly 1979 An improved positive selection plasmid vector constructed by oligonucleotide mediated mutagenesis. *Nucl. Acids Res.*, **11**: 8019-8029.
- Chater, K. F 1989 Multilevel regulation of *Streptomyces* differentiation. *Trends Genet.*, **5**: 372-377.
- Chater, K. F., C. J. Bruton, K. A. Plaskitt, M. J. Buttner, C. Mendez and J. D. Helmann 1989 The development fate of *Streptomyces coelicolor* hyphae depends upon a gene product homologous with the motility sigma factor of *Bacillus subtilis*. *Cell*, **59**: 133-143.
- Chater, K. F., D. A. Hopwood, T. Kieser and C. J. Thompson 1982 Gene cloning in *Streptomyces*. *Curr. Top. Microbiol. Immunol.*, **96**: 69-95.
- Foulger, D. and J. Errington 1989 The role of the sporulation gene *spoIIIE* in the regulation of prespore-specific gene expression in *Bacillus subtilis*. *Mol. Microbiol.*, **3**: 1247-1255.
- Hotta, K., J. Ishikawa, M. Ichihara, H. Naganuma and S. Mizuno 1988 Mechanism of increased kanamycin-resistance generated by protoplast regeneration of *Streptomyces griseus*. I. Cloning of a gene segment directing a high level of an aminoglycoside 3-N - acetyltransferase activity. *J. Antibot.*, **41**: 94-103.
- Kataoka, M., T. Seki and T. Yoshida 1991a Five genes involved in self-transmission of pSN22, a *Streptomyces* plasmid. *J. Bacteriol.*, **173**: 4220-4228.
- Kataoka, M., T. Seki and T. Yoshida 1991b Regulation and function of the *Streptomyces* plasmid pSN22 genes involved in pock formation and inviability. *J. Bacteriol.*, **173**: 7975-7981.
- Kenall, K. J. and S. N. Choen 1987 Plasmid transfer in *Streptomyces lividans*: Identification of *kil* -*kor* system associated with the transfer region of pIJ101. *J. Bacteriol.*, **169**: 4177-4183.
- Kenall, K. J. and S. N. Cohen 1988 Complete nucleotide sequence of the *Streptomyces Zividans* plasmid pIJ101 and correlation of the sequence with genetic properties. *J. Bacteriol.*, **170**: 4634-4651.
- Kieser, T. 1984 Factor affecting the isolation of ccc DNA from *Streptomyces Zividans* and *Escherichia coli*. *Plasmid*, **12**: 19-36.
- Kieser, T., D. A. Hopwood, H. M. Wright and C. J. Thompson 1982 pIJ101, a multi-copy broad host -range *Streptomyces* plasmid: Functional analysis and development of DNA cloning vectors. *Mol. Gen. Genet.*, **185**: 223-238.
- Kyte, J., R. F. Doolittle 1982 A simple method for displaying the hydropathic character a protein. *J. Mol. Biol.*, **157**: 105-132.
- Maniatis, T., E. F. Fritsch and J. Sambrook 1982 "Molecular Cloning : A Laboratory Manual." Cold Spring Harbor Laboratory, New York.
- Miyoshi, Y. K., S. Ogata and S. Hayashida 1986 Multicopy derivative of pock-forming plasmid pSA1 in *Streptomyces azureus*. *J. Bacteriol.*, **168**: 452-454.
- Nakano, M. M., H. Mashiko and H. Ogawara 1984 Cloning of the kanamycin resistance gene from kanamycin-producing *Streptomyces* species. *J. Bacteriol.*, **157**: 79-83.
- Ogata, S., S. Yoshino, H. Suenaga, K. Aoyama, N. Kitajima and S. Hayashida 1981 Specific lysogenicity in *Streptomyces azureus*. *Appl. Environ. Microbiol.*, **42**: 135-141.
- Ogata, S., Y. Koyama, Y. Sakaki and S. Hayashida 1983 Isolation of a linear DNA associated with pock formation in *Streptomyces azureus*. *Agric. Biol. chem.*, **47**: 2127-2129.
- Ogata, S., H. Matsubara, Y. Tawara, S. Tokunaga and T. Hara 1992 Screening of compounds stimulating spore formation and mycelial growth of pock -forming plasmid- carrying strains in *Streptomyces azureus*. *Appl. Microbiol. Biotechnol.*, **37**: 652-654.
- Sanger, F., S. Nicklen and A. R. Coulson 1977 DNA sequencing with chain - terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.*, **74**: 5463-5476.
- Smokvina, T., F. Bocard, J. L. Pernodet, A. Friedmann and M. Guerineau 1991 Functional analysis of the *Streptomyces ambofaciens* element pSAM2. *Plasmid*, **25**: 40-52.
- Stein, D. S., K. J. Kendall and S. N. Cohen 1989 Identification and analysis of transcriptional regulatory signals for the *kil* and *kor* loci of *Streptomyces* plasmid pIJ101. *J. Bacteriol.*, **171**: 5768-5775.
- Szybalski, W. and V. Bryson 1952 Genetic studies on microbial cross resistance to toxic agents.

J. Bacteriol., **64**: 489-488.

Thompson, C. J., J. M. Ward and D. A. Hopwood 1982 Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. *Gene*, 20: 51-62.

Yanisch-Perron, C., J. Vieira and J. Messing 1985 Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. *Gene*, 33: 103-119.