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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

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

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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 (*kmr*) 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-habouring 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-harbouring 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* PKlOOC, 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 μ g/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 PKlOOC 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 PKlOOC 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 PKlOOC against each antibiotics, spore suspension (5×10^2 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 PKlOOC and S. *Zividans* and its derivertives.

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 PK1OOC and PK1OOC(pSA1.l) 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

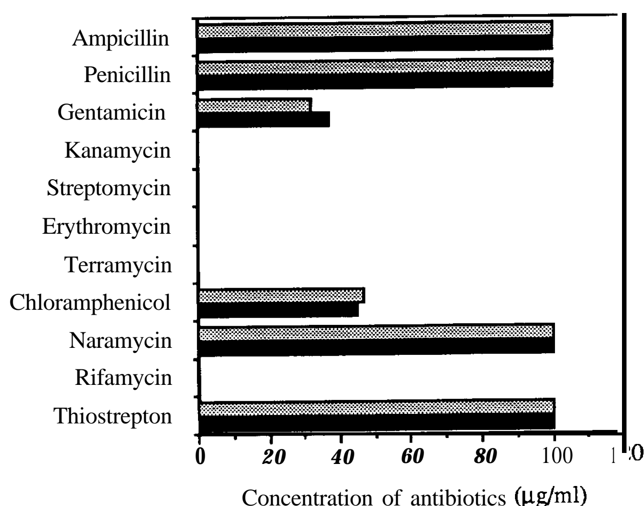

Fig. 1. Resistance of the derivatives of *Streptomyces azureus* ATCC14921 to various antibiotics: ▩, resistibility of strain PK1OOC; ■ , resistibility of strain PK1OOC (pSA1. 1).

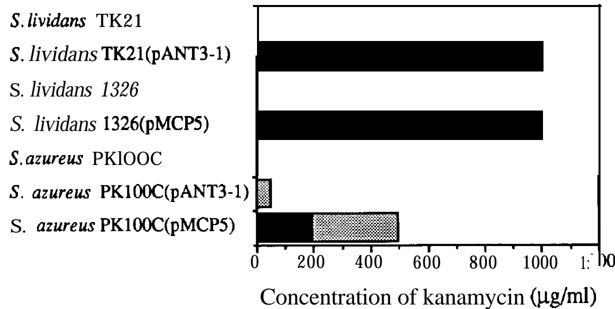

Fig. 2. Kanamycin-resistibility of transformants with *Streptomyces* plasmid vectors, ■,survival rate of 70% or more; ▒,survival rate of 20% to 70%.

these antibiotics were used as selective marker of plasmid pSA1.1 and its host, PK1OOC.

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 PK1OOC 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 PK1OOC (pMCP5) was higher than that of PK1OOC (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 PK1OOC. 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 PK1OOC up to 250 μ g/ml, although the pSA1.1 -harboring PK1OOC was inhibited at less than 5 μ g/ml. And, the kanamycin resistibility of pMCP5 -harboring PK1OOC and pSAK1 -harboring PK1OOC 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 X 10^8 spores/slant and less than 5 X 10^4 spores/slant were produced by strain PK1OOC and by its pSAK1, as reported previously (Ogata ***et al.,*** 1992). The following

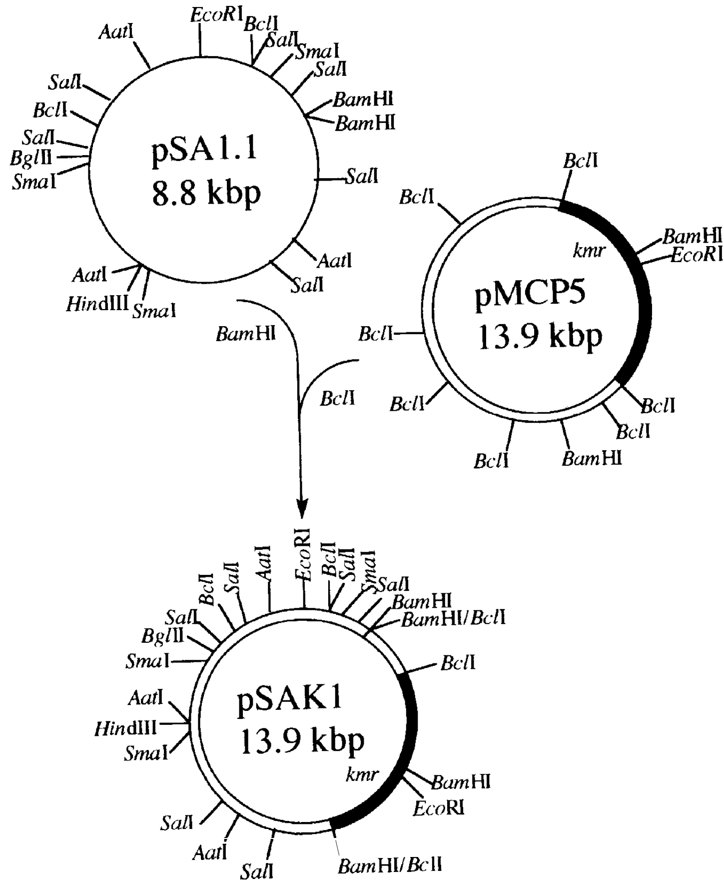

Fig. 3. Construction of plasmid pSAK1 with kanamycin resistant gene (*kmr*)

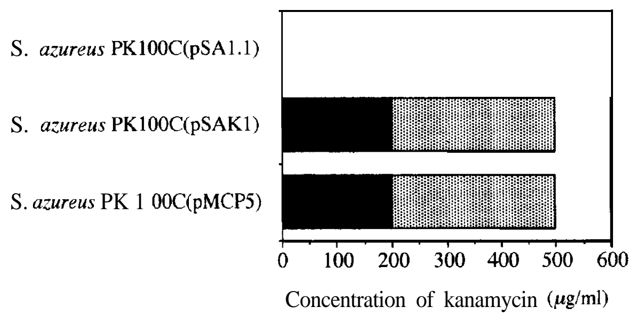

Fig. 4. Kanamycin resistibility of transformants with plasmid pSA1.1 and its derivatives, pSAK1, and the plasmid vector pMCP5. ■ , survival rate of 70% or more; ▒, survival rate of 20% to 70%.

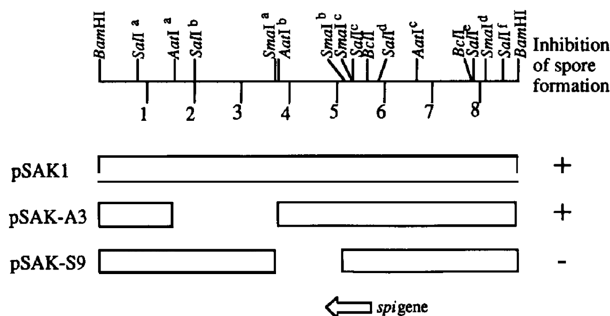

Fig. 5. Delection analysis of pSAK1.
The restriction map of pSA1.1 is shown on the top. The derivatives of pSAK1 are shown as pSAK-A3 and pSAK-S9. They were made by deletion with the appropriate restriction enzymes. +, inhibition of spore formation; −, no inhibition of spore formation. The location and 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 *Aat I"* -*Aat* I^b fragment and 1.6kb *Sma* I^a-*Sma* I^b 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,

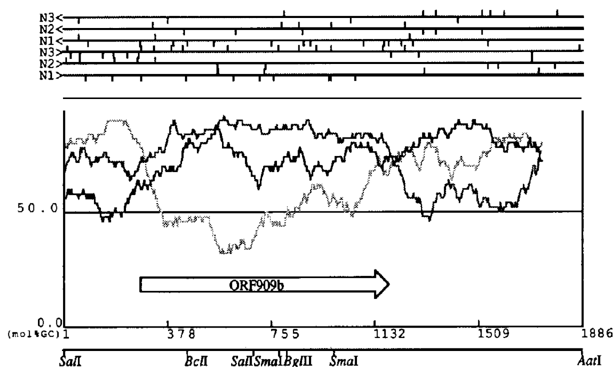

Fig. 6. Frame analysis of *Aat*I^b-*Sal*I^d fragment.
The average of mol% G+C over a 50-codon window is plotted for the first (···), second (.......), and third (_) position in each triplet. The positions of start codon (⊥) and stop codon (⊤) for each possible reading frame (N1>, N2>, N3>, N1<, N2<,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

but pSAK - S9 did not. The number of spores produced by the pSAK - A3 -harboring transformant and by the pSAK-S9-haboring transformant were about 2×10^4 and 8×10^8 (spores/slant), respectively. The deletion of the *Sma* I" -*Sma* I^b fragment led to abundant sporulation. This suggested that a part of or the entire sporulation-inhibitory gene was in the 1.5-kb *Aat* I^b -*Sma* I^b fragment but not in the 2.1 - kb *Aat I"* -*Aat* I^b fragment. This putative gene was named the *spi* gene.

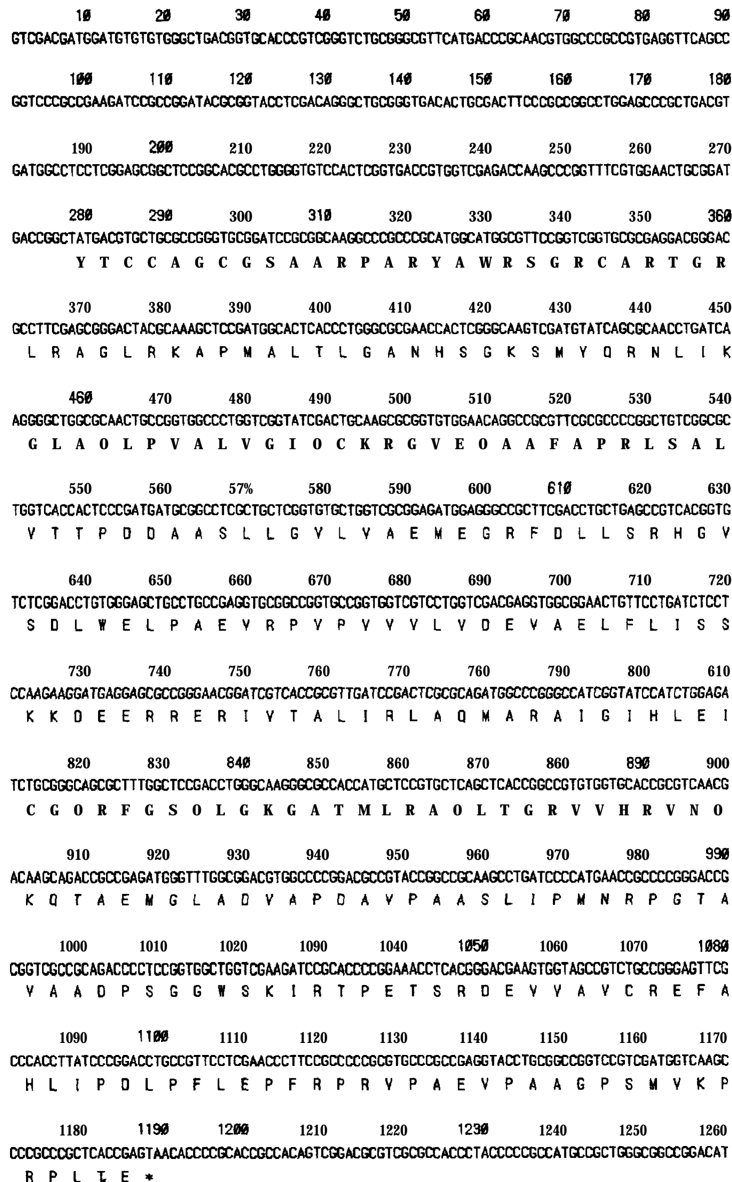

Fig. 7. DNA sequence and predicted amino acid sequence of the *spi* gene.

To examine the pock-forming ability of the two derivative plasmids pSAK-A3 and pSAK-S9, the transformants were spread on Rye flakes agar with the strain PK100C as a recipient. However, none of the transformants could form conjugative pocks. This result suggested that both the 2.1-kb *Aat* I[a]-*Aat* I[b] and 1.6-kb *Sma* I”-Smu I[b] fragments

```
         1                                                                              80
spi      --------------------------------------------------------------------------------

spoIIIE  MAKKKRKSRKKQAKQLNIKYELNGLLCIAISIIAILQLGVVGQTFIYLFRFFAGEWFILCLLGLLVLGVSLFWKKKTPSL

         81                                                                            160
spi      --------------------------------------------------------------------------------

spoIIIE  LTRRKAGLYCIIASILLLSHVQLFKNLTHKGSIESASVVRNTWELFLMDMNGSSASPDLGGGMIGALLFAASHFLFASTG

         161                                                                           240
spi      --------------------------------------------------------------------------------

spoIIIE  SQIMAIVMILIGMILVTGRSLQETLKKWMSPIGRFIKEQWLAFIDDMKSFKSNMQSSKKTKAPSKKQKPARKKQQMEPEP

         241                                                                           320
spi      --------------------------------------------------------------------------------

spoIIIE  PDEEGDYETVSPLIHSEPIISSFSDRNEEEESPVIEKRAEPVSKPLQDIQPETGDQETVSAPPMTFTELENKDYEMPSLD

         321                                                                           400
spi      --------------------------------------------------------------------------------

spoIIIE  LLADPKHTGQQADKKNIYENARKLERTFQSFGVKAKVTQVHLGPAVTKYEVYPDVGVKVSKIVNLSDDLALALAAKDIRI

         401                                                                           480
spi      ----------------------MTCCAGCGSAARPARMAWRSGRCARTGRLRAGLRKAPMALTLGANHSGKSMYQRNL
                                              **   ... *.*.*  *  *. **. ****..
spoIIIE  EAPIPGKSAIGIEVPNAEVAMVSLKEVLESKLNDRPDANVLIGLGRNISGEAVLAELNKMPHLLVAGATGSGKSVCVNGI

         481                                                                           560
spi      IKG----LAQLPVALVGIDCKRGVEQAAF--APR-LSALVTTPDDAASLLGVLVAEMEGRFDLLSRHGVSDL--------
         *..      .  * ..**   *.   **    *. *...**.*..*.  *   .*.*** *..*.*. *....
spoIIIE  ITSILMRAKPHEVKMMMIDPKM-VELNVYNGIPHLLAPVVTDPKKASQALKKVVNEMERRYELFSHTGTRNIEGYNDYIK

         561                                                                           640
spi      ---WELPAEVRPVP-VWVLVDEVAELFLISSKKDEERRERIVTALIRLAQMARAIGIHLEICGQRFGSDLGKGATMLRAQ
             *  *.      .*** .***.*.*....*                 **.*****  ****  *  .** *. .* ...*.
spoIIIE  RANNEEGAKQPELPYIWVIVDELADLMMVAS-------SDVEDSITRLSQMARAAGIHLIIATQRPSVDVITG--VIKAN

         641                                                                           720
spi      LTGRVVHRVNDKQTAEMGLADVAPDAVPAASLIPMNRPGTAVAADPSGGWSKIRTPETSRDEVVAVCREFAHLIPDLPFL
         ...*.. .*... ..   *.          ...          *..    *..     *.    *.*..
spoIIIE  IPSRIAFSVSSQTDSRTILDMGGAEKLLGRGDMLFLPVGANKPVRVQGAFLSDDEVEKVVDHVITQQKAQYQEEMIPEET

         721                                                                      797
spi      EPFRPRVPAEVPAAGPSMVKPRPLTE---------------------------------------------------

spoIIIE  TETHSEVTDELYDEAVELIVGMQTASVSMLQRRFRIGYTRAARLIDAMEERGVVGPYEGSKPREVLLSKEKYDELSS
```

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

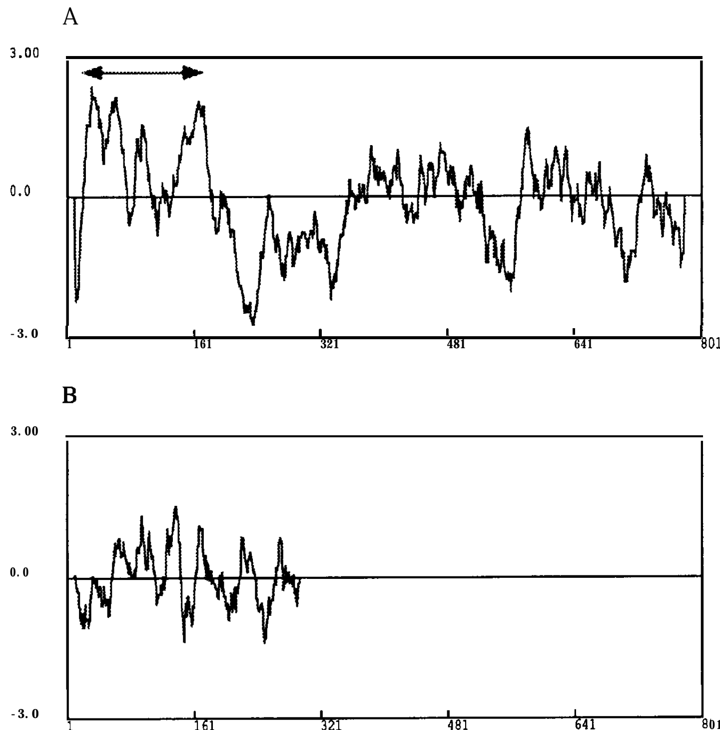

Fig. 9. Hydrophobicity / hydrophilicity plot of *spoIIIE* protein and *spi* protein. The X-axis indicates the amino acid number from the start codon, and Y-axis indicates the relative hydrophobicity / hydrophilicity by method of Kyte and Doolittle. Consecutive hydrophobicity avarages are plotted for a 20-residue window advancing from the NH_3 terminus to COOH terminus. A, *spoIIIE* protein; B, *spi* protein; ⟷ , 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 Doolitle, 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;

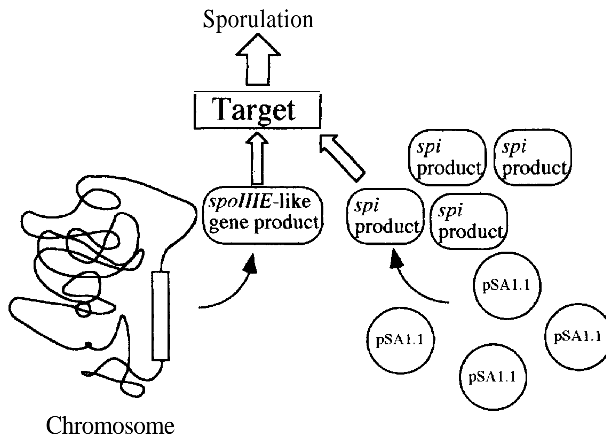

Fig. 10. Schematic model for sporulation-inhibitory function of pSA1.1.

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.

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