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Cloning of sporulation gene *spoIVC* in *Bacillus subtilis*

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Summary. Sporulation gene *spoIVC* of *Bacillus subtilis* was cloned by the prophage transformation method in temperate phage ϕ105. The specialized transducing phage, ϕ105*spoIVC*-1, restored the sporulation of the asporogenous mutant of *B. subtilis* strain 1S47 (*spoIVC133*). Transformation experiments showed that the *spoIVC* gene resides on a 7.3 kb *Hin*dIII restriction fragment. Subsequent analysis of the 7.3 kb *Hin*dIII fragment with restriction endonuclease *Eco*RI showed that the *spoIVC* gene resides on a 3.6 kb *Eco*RI fragment within the 7.3 kb fragment. The 3.6 kb fragment was recloned into the unique *Eco*RI site of plasmid pUB110 and deletion derivatives having a deletion within the 3.6 kb insert were constructed. The plasmid carrying the entire *spoIVC* gene restored the sporulation of strain HU1214 (*spoIVC133, recE4*) at a frequency of 10^7 spores/ml, and reduced the sporulation of strain HU1018 (*spo*$^+$, *recE4*) to 10^7 spores/ml.

Introduction

Extensive information has been accumulated on sporulation in *Bacillus subtilis* and about 50 mutations, blocked at various stages of sporulation, have been identified and mapped (Piggot and Coote 1976). It has been suggested that the expression of the sporulation genes is regulated at the levels of transcription and translation (Sonenshein and Campbell 1978).

The cloning of a sporulation gene makes it possible to clarify the product, function, and organization of the gene. Recently, several sporulation genes have been cloned on phage and plasmid vectors using various systems (reviewed by Lovett 1981), among them, the very useful prophage transformation method. Using this method we have succeeded in cloning the sporulation genes, *spo0F* (Kawamura et al. 1980), *spo0B* (Hirochika et al. 1981), *spoIIC* (Anaguchi et al. 1984b), *spoIIG* (Ayaki and Kobayashi 1984) and *spoVE* (Yamada et al. 1983) on *B. subtilis* phage vectors. The cloned genes were recloned from the specialized transducing phages to a multicopy plasmid pUB110 for detailed analysis. Recombinant plasmids carrying the sporulation gene *spo0F, spoIIG,* or *spoVE* inhibit the sporulation of a wild-type strain, while those carrying the *spo0B* gene have no effect on sporulation. The present paper describes the cloning of the sporulation gene *spoIVC* of *B. subtilis* in the temperate bacteriophage ϕ105 using the prophage transformation method, and some characteristics of the cloned gene.

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Materials and methods

Bacterial and phage strains. The bacterial strains used in this study are listed in Table 1. Strain 61242 was used as the indicator for plaque-forming unit assays. Temperate phage ϕ105 (Rutberg 1969) was obtained from H. Saito. Phage ϕ105c (Shibata and Ando 1974) is a clear plaque mutant of ϕ105. Defective phage PBSX (Okamoto et al. 1968) was induced from a spore-forming strain JH642 by mitomycin C treatment and its DNA was used as a source of *B. subtilis* chromosomal DNA in the prophage transformation experiment (Kawamura et al. 1979).

Table 1. *Bacillus subtilis* strains

Strain	Genetic character	Origin or source
JH642	*trpC2 pheA1*	J. Hoch
1S47	*trpC2 spoIVC133*	*Bacillus* Genetic Stock Center (Ohio State University)
61242	*cysB3 trpC2 hisA1*	University of Tokyo
UOT0277	*hisA1 metB5 nonB1 recE4*	University of Tokyo
UOT0531	*trpC2 metB51 leuA8 nonB1*	University of Tokyo
HU1018	*trpC2 metB51 nonB1 recE4*	*recE4* transformed from UOT0277 into UOT0531 by congression with *leu*$^+$
HU1122	*trpC2 metB51 leuA8 nonB1 spoIVC133*	*spoIVC133* transformed from 1S47 into UOT0531
HU1214	*trpC2 metB51 nonB1 recE4 spoIVC133*	*recE4* transformed from UOT0277 into HU1122 by congression with *leu*$^+$
1S47 (ϕ105)	1S47 lysogenic for ϕ105	This laboratory

Preparation of phage and phage DNA. Preparation of phage lysates and phage DNA from lysogenic strains was performed as described before (Yamada et al. 1983).

Construction of specialized transducing phages φ105spoIVC-1 and φ105spoIVC-2. Specialized transducing phage φ105*spoIVC*-1 was prepared by cloning *Hin*dIII fragments of *B. subtilis* DNA into φ105 by the prophage transformation method as described previously (Hirochika et al. 1981), except that PBSX DNA and φ105 DNA were digested with restriction endonuclease *Hin*dIII and strain 1S47 (*spoIVC133*), lysogenic for φ105, was used as the recipient strain. For the construction of φ105*spoIVC*-2 from φ105-*spoIVC*-1 the *Hin*dIII fragment cloned in φ105*spoIVC*-1 was isolated and cleaved into three fragments with restriction endonuclease *Eco*RI. Then, the 3.6 kb *Eco*RI fragment having *Spo*$^+$ transforming activity for strain 1S47 was recloned in φ105 by the prophage transformation method, using restriction endonuclease *Eco*RI. A specialized transducing phage φ105*spoIVC*-2 having the 3.6 kb *Eco*RI fragment was isolated from the Spo$^+$ transformants. Restriction enzyme analysis, DNA ligation, and isolation of DNA fragments were performed as described previously (Yamada et al. 1983).

Preparation of plasmid DNA. Plasmid pUB110 was used as a cloning vector (Gryczan et al. 1978). Selection of recombinant plasmids and deletion plasmids by analysis on agarose gel electrophoresis and purification of plasmid DNA were performed as described before (Yamada et al. 1983).

Transformation. Bacterial transformation was performed by the method of Wilson and Bott (1968) and Spo$^+$ transformants were selected as previously described (Hoch 1971).

Enzymes and chemicals. Lysozyme and RNase A were purchased from the Sigma Chemical Co., St. Louis, Mo, USA. All the restriction enzymes and T4 DNA ligase were purchased from the Nippon Gene Co. Ltd., Japan. Agarose was purchased from the Takara Shuzo Co. Ltd., Japan.

Results and discussion

Construction and characterization of φ105spoIVC-1

Using *B. subtilis* temperate phage φ105, specialized transducing phage φ105*spoIVC*-1 was constructed by the prophage transformation method (Kawamura et al. 1979; Iijima et al. 1980). This phage, when lysogenized in the asporogenous mutant 1S47 (*spoIVC133*), restores the sporulating ability of the mutant completely (10^8 spores/ml). This suggests that the *spoIVC133* mutation is recessive to the wild-type allele and that the cloned *spoIVC* gene functions normally in the lysogen. The specialized transducing phage was induced with mitomycin C from the Spo$^+$ transformants and assayed for transducing activity (Table 2). The lysate had no plaque-forming ability, but exhibited transducing activity when helper phage (φ105) was added. If the helper phage was omitted, transductants were not obtained, whereas DNA extracted from this specialized transducing phage showed Spo$^+$ transforming activity. These results suggest that φ105*spoIVC*-1 does not require the helper phage for induction, but does for lysogenization. Similar results were obtained with other φ105 specialized transducing phages (Hirochika et al. 1982; Yamada et al. 1983; Anaguchi et al. 1984b). Therefore, it is likely that the φ105 transducing phage obtained here lacks a tail as demonstrated by Jenkinson and Mandelstam (1983).

Table 2. The *spoIVC* transducing activity of φ105*spoIVC*-1 and φ105*spoIVC*-2

Phages	Recipient	Number of Spo$^+$ transductants/ml
φ105*spoIVC*-1	1S47	<10
φ105*spoIVC*-1 + φ105	1S47	6.1×10^7
φ105*spoIVC*-2	1S47	1.2×10^2
φ105*spoIVC*-2 + φ105	1S47	1.9×10^7
φ105	1S47	<10
None	1S47	<10

Phage lysate (0.5 ml) was added to 4.5 ml of 1S47 cells (2×10^7 cells/ml) in Schaeffer sporulation medium (Schaeffer et al. 1965). Helper phage φ105 was added at a multiplicity of infection of 5. Cells were incubated for 24 h at 37° C. Heat-resistant spores were counted by plating the cells on NB plates (8 g of Difco nutrient broth, 15 g of agar, and 1 l of water) after heating the cells for 10 min at 80° C

Identification of the HindIII fragment carrying the spoIVC gene

A *Hin*dIII fragment carrying the *spoIVC* gene was identified by transformation of φ105*spoIVC*-1 DNA digested with *Hin*dIII. The DNA fragments obtained from φ105*spoIVC*-1 DNA were analysed by agarose gel electrophoresis. New 7.3 kb and 1.7 kb fragments appeared in the φ105*spoIVC*-1 genome, whereas a 4.7 kb fragment disappeared (Fig. 1). Transformation experiments using DNA fragments purified from low-melting-point agarose gels showed that the *spoIVC*$^+$ transforming activity is carried by the 7.3 kb fragment (data not shown).

Subsequent analysis showed that the *spoIVC* gene resides on a 3.6 kb *Eco*RI fragment within the 7.3 kb *Hin*dIII fragment. The 7.3 kb *Hin*dIII fragment has two *Eco*RI sites, and 1.7, 2.0 and 3.6 kb fragments were generated by *Eco*RI treatment. These fragments were purified by agarose gel electrophoresis and tested for *spoIVC*$^+$ transforming activity. Among them the 3.6 kb *Eco*RI fragment had Spo$^+$ transforming activity (data not shown). Subsequently, the 3.6 kb *Eco*RI fragment was subcloned in φ105 by the prophage transformation method. The resulting specialized transducing phage was designated as φ105*spoIVC*-2. As shown in Table 2, φ105*spoIVC*-2 had the same characteristics as φ105*spoIVC*-1 and showed no plaque-forming activity in the absence of the helper phage φ105. Agarose gel electrophoresis of the *Eco*RI-digested, φ105*spoIVC*-2 DNA showed that φ105*spoIVC*-2 has the 3.6 kb *Eco*RI fragment, but lacks the *Eco*RI G and I fragments which are present in the φ105 genome (Fig. 1).

Construction of restriction maps of φ105spoIVC-2 DNA and the 3.6 kb fragment

To determine the location of the 3.6 kb fragment in the φ105*spoIVC*-2 genome, an *Eco*RI restriction map was constructed with reference to that of the φ105 genome (Anagu-

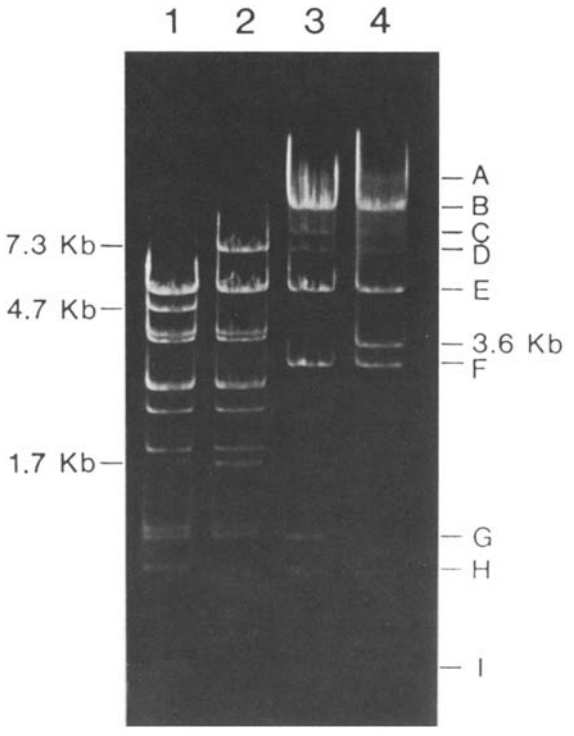

Fig. 1. Agarose gel electrophoresis of *Hin*dIII- or *Eco*RI-digested DNA fragments of ϕ105*spoIVC*-1 and ϕ105*spoIVC*-2. *Hin*dIII- or *Eco*RI-digested DNA (1 μg) was analysed by agarose gel electrophoresis. The size of the fragments in kb was determined from their electrophoretic mobilities relative to λ*Hin*dIII fragments. Fragment A consists of terminal fragments C and D. 1, *Hin*dIII cleavage fragments of ϕ105; 2, *Hin*dIII cleavage fragments of ϕ105*spoIVC*-1; 3, *Eco*RI cleavage fragments of ϕ105; 4, *Eco*RI cleavage fragments of ϕ105 *spoIVC*-2

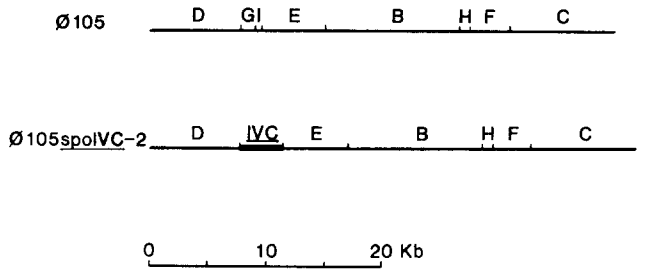

Fig. 2. *Eco*RI cleavage map of ϕ105 and ϕ105*spoIVC*-2 DNA. The cleavage map of ϕ105 DNA was taken from Anaguchi et al. (1984a). The *thick line* on the ϕ105*spoIVC*-2 DNA indicates the cloned 3.6 kb fragment

chi et al. 1984a). The arrangement of the fragments was determined by *Eco*RI or *Eco*RI and *Sma*I double digestion of the phage DNA (data not shown). The results are summarized in Fig. 2. The genome of ϕ105*spoIVC*-2 lacks *Eco*RI fragments G and I which are present in the ϕ105 genome. The 3.6 kb fragment is inserted between the *Eco*RI fragments D and E suggesting that during prophage transformation a double crossing-over occurred in fragments D and E. In all ϕ105 specialized transducing phages so far constructed (Iijima et al. 1980; Hirochika et al. 1981; Jenkinson and Mandelstam 1983; Yamada et al. 1983; Anaguchi et al. 1984b; Ayaki and Kobayashi 1984), the cloned

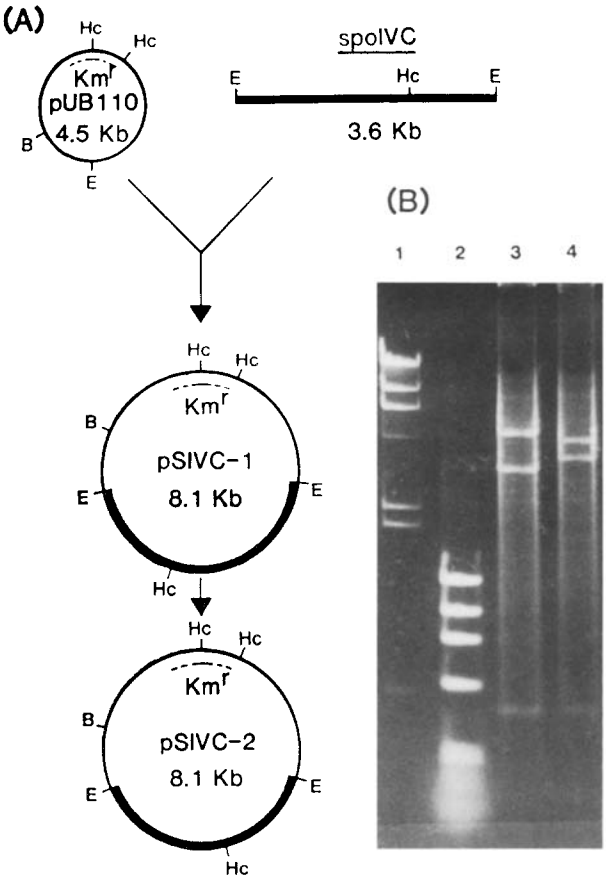

Fig. 3A, B. Construction of pSIVC-1 and pSIVC-2, and agarose gel electrophoresis of *Hin*cII cleavage fragments of pSIVC-1 and pSIVC-2. **A** Construction of pSIVC-1 and pSIVC-2. pSIVC-1 is a recombinant plasmid carrying the 3.6 kb *Eco*RI fragment at the unique *Eco*RI site of pUB110; pSIVC-2 has the 3.6 kb *Eco*RI fragment in the opposite orientation. Restriction enzymes: Hc, *Hin*cII; B, *Bam*HI; E, *Eco*RI. **B** Agarose gel electrophoresis of *Hin*cII digests of pSIVC-1 and pSIVC-2. The fragments were separated on a 0.8% agarose gel. 1, *Hin*dIII-digested λDNA; 2, *Hae*III-digested ϕX174 DNA; 3, *Hin*cII-digested pSIVC-2; 4, *Hin*cII-digested pSIVC-1

fragment was inserted in the region of the *Eco*RI fragments G, I, and E.

Construction of pSIVC and its deletion derivatives

In contrast to the phage vector, which exists as a single prophage genome in the host cell, plasmid vectors such as pUB110 are known to exist in multiple copies. The copy-number effect of sporulation genes on *B. subtilis* has been reported (Kawamura et al. 1981). To examine the copy-number effect of the *spoIVC* gene on the sporulation process, a recombinant plasmid was constructed with plasmid pUB110 and the *spoIVC* gene as described previously (Yamada et al. 1983; Fig. 3A). To screen for recombinant plasmids carrying the cloned insert, the plasmid DNAs prepared from 200 purified Km^r colonies were analysed by agarose gel electrophoresis. Two recombinants were found and restriction analysis and transformation experiments showed that these plasmids, designated as pSIVC-1, carried the 3.6 kb insert in pUB110.

To obtain a plasmid having the 3.6 kb fragment inserted

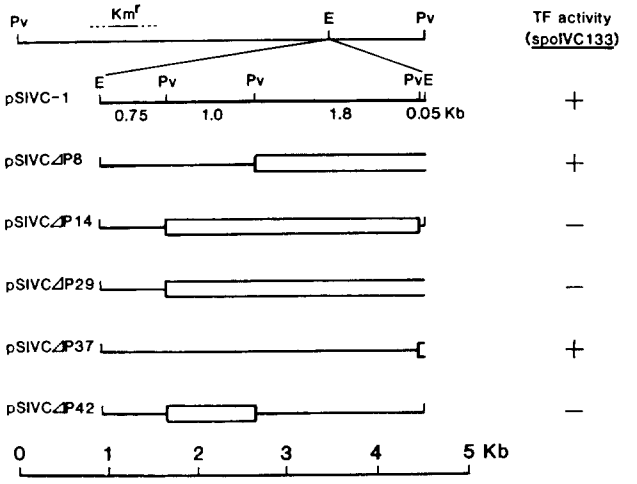

Fig. 4. *Pvu*II cleavage map of the 3.6 kb fragment and deletion derivatives of the plasmid pSIVC. *Upper part:* the top line shows the pUB110 DNA, the second line the restriction map of the 3.6 kb fragment. Restriction enzymes: E, *Eco*RI; Pv, *Pvu*II. The sizes of the fragments are indicated in kilobases. *Lower part:* location of the deletions in the 3.6 kb insert of the plasmid pSIVC. The *line* shows the 3.6 kb insert of the plasmids. Deleted portions were indicated by *boxes*. The transforming activity (TF) of the deletion plasmids is shown at the right with ability to transform strain HU1122 (*spoIVC133*) to Spo$^+$ shown by +

in the opposite orientation, pSIVC-1 was treated with *Eco*RI, ligated with T4 DNA ligase and used to transform strain HU1214 to Km^r. One of these Km^r transformants contained a plasmid carrying the 3.6 kb fragment in the opposite orientation, since this plasmid generates three *Hin*cII fragments whose sizes coincide with those expected for such an orientation (Fig. 3B). This plasmid was designated as pSIVC-2.

To determine the location of the *spoIVC* gene in the 3.6 kb fragment, we tried to construct plasmids having a deletion in the 3.6 kb insert of pSIVC. There are three *Pvu*II restriction sites in the cloned fragment and one in the vector pUB110 (Fig. 4). Deletion plasmids were constructed by partially digesting pSIVC-1 with *Pvu*II and religating the restricted DNAs. The ligated mixture was used to transform strain HU1018 to Km^r. The Km^r transformants were purified and the deletion plasmids (whose DNA molecular weight is smaller than that of pSIVC) were screened for by agarose gel electrophoresis. Five deletion plasmids isolated were purified and analysed by restriction enzyme digestion. They were designated as pSIVCΔP8, pSIVCΔP14, pSIVCΔP29, pSIVCΔP37, and pSIVCΔP42, respectively. Transformation with the deletion derivatives of pSIVC-1 was performed using HU1122 (*spoIVC133*) as the recipient strain. As shown in Fig. 4, pSIVCΔP8 and pSIVCΔP37 showed *spoIVC133*$^+$ transforming activity, but no transforming activity was found with the other derivatives. These results suggest that the *spoIVC133* marker is located in the 1.0 kb *Pvu*II fragment.

Gene dosage effect on sporulation by pSIVC and its deletion derivatives

To test the sporulating ability of Spo$^-$ strains harbouring pSIVC-1, pSIVC-2, or the deletion plasmids, each plasmid was transferred into an *spoIVC*$^-$ recipient carrying the *recE4* mutation (HU1214). The results are shown in Table 3; pSIVC-1, pSIVC-2, and pSIVCΔP37 complemented the *spoIVC133* mutation and pSIVCΔP8 slightly restored the sporulation of HU1214, but pSIVCΔP14, pSIVCΔP29, and pSIVCΔP42 did not complement the *spoIVC133* mutation. These results indicate that the cloned *spoIVC* gene is functional in both orientations and presumably carries its own promoter and structural gene.

Table 3. Sporulation of *spoIVC* and *spo*$^+$ strains harbouring pSIVC derivatives

Host cell	Plasmid	Viable cells/ml	Spores/ml
HU1214 (*spoIVC133, recE4*)	–	2.9×10^8	<10
	pUB110	1.8×10^8	<10
	pSIVC-1	3.8×10^8	2.4×10^7
	pSIVC-2	4.4×10^8	7.8×10^7
	pSIVCΔP8	3.0×10^8	5.7×10^3
	pSIVCΔP14	3.8×10^8	<10
	pSIVCΔP29	3.4×10^8	<10
	pSIVCΔP37	3.0×10^8	1.6×10^6
	pSIVCΔP42	3.5×10^8	<10
HU1018 (*spo*$^+$, *recE4*)	–	1.5×10^8	1.4×10^8
	pUB110	1.5×10^8	1.2×10^8
	pSIVC-1	1.6×10^8	5.4×10^7
	pSIVC-2	1.1×10^8	6.5×10^7
	pSIVCΔP8	1.5×10^8	1.3×10^8
	pSIVCΔP14	1.6×10^8	1.7×10^8
	pSIVCΔP29	1.3×10^8	1.6×10^8
	pSIVCΔP37	1.4×10^8	7.4×10^7
	pSIVCΔP42	1.3×10^8	6.0×10^6

The plasmid-harbouring strains were inoculated into 5 ml of Schaeffer sporulation medium (Schaeffer et al. 1965) supplemented with kanamycin at 5 μg/ml (for the strain lacking a plasmid, Km was omitted). Viable cells were counted by plating the cells on NB plates at 5 h after the end of exponential growth. Heat-resistant spores were counted as described in Table 2

Previously, we had observed an inhibitory effect on the sporulation of a wild-type (Spo$^+$) strain by recombinant plasmids carrying the *spoOF* (Kawamura et al. 1981), *spoIIC* (Anaguchi et al. 1984b), *spoIIG* (Ayaki and Kobayashi 1984), and *spoVE* (Yamada et al. 1983) genes. Therefore, we tested the effect of the pSIVC and the deletion plasmids on the sporulation of the wild-type strain by transferring pSIVC-1, pSIVC-2, and the deletion plasmids into an Spo$^+$ recipient carrying the *recE4* mutation (HU1018). As shown in Table 3, pSIVC-1, pSIVC-2, pSIVCΔP37, and pSIVCΔP42 slightly inhibited sporulation, but the other deletion plasmids and pUB110 did not do so. If the inhibition of sporulation is caused by the promoter-titration of RNA polymerase (reviewed by Losick 1982) it is possible that the promoter region of the *spoIVC* gene may be to the right of the *spoIVC133* mutation but if the slight restoration of sporulation by pSIVCΔP8 is caused by the *spoIVC* gene product then the promoter region of the *spoIVC* gene may be to the left. Further analysis is necessary to reach a final conclusion.

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