Isolation and Characterization of a Clone of the *spoVE* Locus of *Bacillus subtilis*

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The *Bacillus subtilis spoVE* locus was isolated from a λ clone bank and a 4.7 kbp *EcoRV* fragment subcloned into the shuttle vector pHV33. The resulting plasmid complemented chromosomal *spoVE* mutations. Its structure was stable in *recE4* strains, but plasmid and chromosomal rearrangements occurred in *rec+* strains. New *spoVE* mutations were obtained by mutagenesis of the plasmid; all the mutations tested mapped within three adjacent *HindIII* fragments of total length 1140 bp.

INTRODUCTION

Sporulation of *Bacillus subtilis* is a primitive system of cellular differentiation. At the start of the process, there is a single (vegetative) type of cell. This cell divides asymmetrically. The smaller cell that results, the forespore, is then gradually engulfed by the larger cell, the mother cell. These two cell types coexist for several hours, and both are necessary for the forespore to develop into the mature spore. At, or soon after, the completion of spore maturation, the mother cell lyses. Nearly 50 genetic loci thought to be specifically involved in spore formation have been identified by mutations (called *spo* mutations) that block sporulation but do not impair vegetative growth (Piggot & Coote, 1976). Significant advances in our understanding of the regulation of *spo* locus expression have been made by studies of cloned *spo* loci (Losick & Youngman, 1984; Piggot, 1985). These studies have concentrated on loci involved in the early part of sporulation. We are interested in analysing the control of gene expression during later stages, when the mother cell and forespore have been formed. To this end, we are interested in studying clones of loci whose expression is thought to be specific to each of the two cell types (Lencastre & Piggot, 1979). The *spoVA* locus, thought to be expressed in the forespore, has been cloned (Piggot *et al.*, 1984; Savva & Mandelstam, 1984) and sequenced (Fort & Errington, 1985). Here, we report the cloning of the *spoVE* locus, which is thought to be expressed in the mother cell (Lencastre & Piggot, 1979).

METHODS

Strains. The *B. subtilis* 168 strains used are listed in Table 1. The *Escherichia coli* strains used were ED8767 (*supE supF recA56 HsdS⁻ Met⁻*; Murray *et al.*, 1977); DH1 (*endA1 hsdR17* [Res⁻(K) Mod⁺(K)] *supE44 thi-1 recA1 gyrA96 relA1*; Hanahan 1983); SL2034, a Dam⁻ strain whose origin is obscure; and SL3617 (SL2034 transformed with pPP7, see text). Plasmids were maintained in DH1 or SL2034 when *BclI* was used, unless otherwise stated. The plasmid vectors used were pHV33 (Primrose & Ehrlich, 1981) and pJAB1 (Sargent & Bennett, 1985).

Identification of Charon 4A clones containing *spoVE* DNA. One thousand isolated plaques from the Charon 4A bank of Ferrari *et al.* (1981) were picked, suspended in λ buffer and arranged in a three-dimensional matrix with

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Table 1. *B. subtilis* strains

Designation	Genotype/phenotype	Origin*
GSY289	<i>pyrD1 trpC2</i>	C. Anagnostopoulos
GSY908	<i>argC4 hisA1 recE4</i>	C. Anagnostopoulos
SL14	<i>spoIIIE36 trpC2</i>	Laboratory stock
SL54	<i>spoIIIE24 trpC2</i>	DNA 24T (P. Schaeffer) × SL10 (Lencastre & Piggot, 1979) → Phe ⁺
SL617	<i>spoIIG55 leuA8 tal-1</i>	Laboratory stock
SL666	<i>spoVE85 metC3 tal-1</i>	DNA 85 (Hranueli <i>et al.</i> , 1974) × MB21 (Piggot & Taylor, 1977) → Leu ⁺
SL761	<i>spoOE160 trpC2</i>	Laboratory stock
SL931	<i>spoVD156 trpC2 rif-2</i>	Laboratory stock
SL1022	NonA ⁻ NonB ⁻ (HsrM ⁻) <i>leu-8</i>	Y12S (Saito <i>et al.</i> , 1979)
SL1093	NonA ⁻ HsrM ⁻ <i>arg-15 recE4</i>	DNA GSY908 × SL1022 → Leu ⁺
SL1094	NonA ⁻ HsrM ⁻ <i>leu-8 spoVE153</i>	DNA W5 (Coote, 1972) × SL1022 → Arg ⁺
SL1095	NonA ⁻ HsrM ⁻ <i>leu-8 spoVE85</i>	DNA SL666 × SL1022 → Arg ⁺
SL1097	NonA ⁻ HsrM ⁻ <i>spoVE85 recE4</i>	DNA GSY908 × SL1095 → Leu ⁺
MB75	<i>metC3 lys-1 tal-1</i>	Laboratory stock
MT1	<i>metC3 spoVE1 tal-1</i>	This paper
MT2	<i>metC3 spoVE2 tal-1</i>	This paper
MT5	<i>metC3 spoVE5 tal-1</i>	This paper
MT8	<i>metC3 spoVE8 tal-1</i>	This paper
BR151	<i>metB10 lys-3 trpC2</i>	R. S. Buxton

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axes *x*, *y* and *z* numbered 0 to 9. For each point on the *x* axis, all the plaques in the other two dimensions were pooled (i.e. 100 clones). The procedure was repeated for the *y* and *z* axes, giving 30 pools (Monteiro *et al.*, 1984). After amplification (DeWet *et al.*, 1980), the phage DNA was prepared and screened for its ability to transform SL666 to Spo⁺. Eight of the *x* pools, four of the *y* pools, and three of the *z* pools had substantial activity, indicating that abundant *spoVE*⁺ clones were present. Using this information, ten positive clones were isolated; no attempt was made to isolate all the positive clones that might have been present.

In general, phage were handled as described by Monteiro *et al.* (1984).

Construction of pPP7. An *EcoRV* fragment of 4.7 kbp from phage 732 had *spoVE85*⁺ transforming activity, and was separated from other fragments by agarose gel electrophoresis and purified by the method of Dretzen *et al.* (1981). It was ligated to pHV33 that had been digested with *EcoRV*; pHV33 has a single *EcoRV* site within the determinant of tetracycline resistance. The ligated mixture was used to transform ED8767 to ampicillin resistance. Plasmid was prepared from the lawn of transformants obtained, and used to transform *B. subtilis* SL666 to chloramphenicol resistance. Six of 1052 such transformants were Spo⁺, and from each of these, plasmid DNA was prepared and used to transform *E. coli* SL2034 to ampicillin resistance. With five of the donors, the SL2034 transformants were tetracycline sensitive, suggesting insertion at the *EcoRV* site of pHV33; plasmid prepared from 12 such transformants had the size and transforming activity expected. One transformant, SL3617, with a plasmid designated pPP7, was used for further study.

Sporulation. Bacteria were induced to sporulate in liquid culture using Schaeffer's sporulation medium as modified by Leighton & Doi (1971), except that cultures contained 5 µg chloramphenicol ml⁻¹. Viable counts were determined for cultures 18 h after the end of exponential growth by diluting cultures in Spizizen (1958) minimal salts containing 0.5% glucose and by plating them on nutrient agar. Spore counts at this time were determined by heating cultures to 85 °C for 20 min and then diluting and plating.

Mutagenesis of plasmid pPP7. Plasmid DNA was treated with *o*-methylhydroxylamine as described by Bresler *et al.* (1983) except that a 0.75 M solution was used. Mutants were tentatively identified as SpoV by the reddish-brown pigmentation of their colonies on nutrient agar. This was confirmed by examination with a phase contrast microscope, where SpoV mutants have a characteristic appearance (Piggot, 1978).

Media. In general, *B. subtilis* strains were maintained on nutrient agar and *E. coli* strains maintained on L agar. Appropriate antibiotics were added, at the following concentrations, for strains harbouring plasmids: chloramphenicol (Cm), 5 µg ml⁻¹; tetracycline, 20 µg ml⁻¹; ampicillin, 25 µg ml⁻¹. L agar (pH adjusted to 7.0) contained, per litre: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g; Difco agar, 16 g. Nutrient agar consisted of Difco nutrient broth solidified by 1.6% (w/v) Oxoid Bacteriological agar. Modified Schaeffer's sporulation medium (pH adjusted to 7.0) contained, per litre: Difco nutrient broth, 16 g; MgSO₄·7H₂O, 0.5 g; KCl, 2 g. This was freshly prepared, or re-steamed prior to use, as better sporulation was obtained by these

procedures. $\text{Ca}(\text{NO}_3)_2$ (to 10^{-3} M), MnCl_2 (10^{-4} M), FeSO_4 (10^{-6} M) and glucose (0.1%, w/v) were added before use (Leighton & Doi, 1971).

Transformation. Spo^+ (chloroform-resistant) transformants were selected by the method of Hoch (1971). Transformation was done as described by Piggot *et al.* (1984).

Other methods. All other methods have been described previously (Piggot *et al.*, 1984).

RESULTS AND DISCUSSION

Isolation of Charon 4A clones containing spoVE⁺ transforming activity

Ten *spoVE*⁺ clones were identified and isolated from the Charon 4A bank of Ferrari *et al.* (1981). Each positive clone was reisolated twice from single plaques, and DNA was prepared from the phage. All gave the same restriction pattern on digestion with *EcoRI*, and so one isolate, 732, was used for further analysis. None of a series of strains with genetic markers located near to *spoVE* on the genetic map, namely SL931 *spoVD156*, SL617 *spoIIG55*, SL14 *spoIIIE36*, SL54 *spoIIIE24*, SL761 *spoOE160*, GSY289 *pyrD1*, gave wild-type recombinants with 732 DNA.

The restriction map of 732 DNA is shown in Fig. 1. The insert consisted of two *EcoRI* fragments of 2.8 and 10.5 kbp. These were identical in size to fragments of *B. subtilis* chromosomal DNA digested with *EcoRI* that hybridized to a probe derived from 732 DNA (pPP7, see below) on Southern (1979) blots, indicating that there had been no rearrangement on cloning.

The *spoVE85*⁺ transforming activity was located in the right end of the insert (in the sense of Fig. 1), as it was present in the 9.5 kbp *BamHI* fragment spanning this region. Neither of the *EcoRI* fragments of the insert were active in such a transformation, suggesting either that the *spoVE85* mutation was near an *EcoRI* site or that the 2.8 kbp fragment was too small to be active in transformation (data not shown).

Construction of a plasmid that complements spoVE mutations

It was established that a 4.7 kbp *EcoRV* fragment of 732 had *spoVE85*⁺ transforming activity. This fragment was isolated and subcloned into the single *EcoRV* site of pHV33 to produce pPP7, whose restriction map is shown in Fig. 2. The *EcoRV* fragments derived by restriction of pPP7 were identical in size to pHV33 cut with *EcoRV* and to the 4.7 kbp fragment derived from 732; the 2.8 kbp *EcoRI* fragment from the insert in pPP7 was identical in size to the 732 fragment. This indicates that there was no rearrangement on subcloning. The map confirms and extends that of Yamada *et al.* (1983) for an independently isolated clone of *spoVE*. It should be noted that a portion of the cloned DNA in pPP7 was from the vector Charon 4A rather than *B. subtilis*; a complete map of *EcoRV* sites on 732 was not determined.

Complementation of chromosomal spoVE mutations by pPP7

B. subtilis SL1097 (*spoVE85 recE4* HsrM⁻) was transformed to Cm^R with pPP7 and with pHV33. All the pPP7 transformant clones were dark brown, and all those examined by phase contrast microscopy were Spo^+ . All pHV33 transformant clones were red-brown (characteristic of stage V mutants), and all those examined by phase contrast microscopy were blocked at stage V of sporulation (SpoV). Subculturing the pPP7 transformants in the absence of chloramphenicol led to the rapid segregation out of Cm^S SpoV bacteria. From this it was deduced that the cloned DNA on pPP7 functioned to complement the chromosomal *spoVE85* mutation. Similar complementation was observed for the *spoVE153* mutation. Transformation of SL1093 (*recE4* Spo^+ HsrM⁻) with pPP7 and with pHV33 gave Cm^R transformants that were Spo^+ as judged by observation of colony pigmentation and by phase-contrast microscopy. Thus *spoVE* did not show the inhibition of sporulation when present in multiple copies that has been noted for *spoOF* (Kawamura *et al.*, 1981) and *spoVG* (Banner *et al.*, 1983).

After the completion of the above experiments, Yamada *et al.* (1983) reported the cloning in the vector $\phi 105$ of an *EcoRI* fragment of about 2.6 kbp that could complement the *spoVE85* mutation. Their *HindIII* restriction map of this fragment agreed with ours. In contrast to our

Fig. 1. Restriction map of phage 732. The insert of *B. subtilis* DNA within the Charon 4A arms is hatched; it is bounded by *EcoRI* sites. The map of *EcoRV* sites is incomplete; only those used to make pPP7 are shown.

Fig. 2. Restriction map of pPP7. The insert from phage 732 is shown as a linear map, the vector, pHV33, as a circle. The part of the insert derived from Charon 4A is stippled. The insert is drawn in the opposite direction to that in Fig. 1 so that the direction of reading of the *spoVE* open reading frame, indicated by the arrow, is from left to right (see Bugaichuk & Piggot, 1986). The map also shows the *HindIII* fragments subcloned in pJAB1 to create pPP73, pPP74, pPP75 and pPP76.

results, however, they reported that a subclone of the *EcoRI* fragment on the *B. subtilis* plasmid vector pUB110 inhibited sporulation of a *recE4* *Spo*⁺ strain. We therefore examined the effect of pPP7 on sporulation in liquid culture (Table 2). The sporulation of SL1097 (*spoVE85*/pPP7) was good, 28%, although not as good as that of the strain with *spo*⁺ chromosome (SL1093). Neither pPP7 nor pHV33 impaired the sporulation of the strain with a *spo*⁺ chromosome, although with pPP7 the proportion of spores able to germinate and grow when chloramphenicol was added (presumably those harbouring plasmid) was greatly reduced (Table 2). This may indicate that pPP7 is readily lost even in the presence of chloramphenicol, and only when there is also a requirement for the *spoVE*⁺ function of the plasmid do the majority of sporulating organisms retain the plasmid. These studies confirm the plate tests, showing that pPP7 complements chromosomal *spoVE* mutations efficiently and does not inhibit sporulation of a *Spo*⁺ strain. The most probable explanation for this difference between our results and those of Yamada *et al.* (1983) is that they used pUB110 as vector whilst we used pHV33. The difference may simply be a matter of copy number. pUB110 is reported to be present at about 40 copies per chromosome (Scheer-Abramowitz *et al.*, 1981) and recombinant plasmids derived from pUB110 may have similar values. When pHV33 is vector, copy numbers are in the range 10 to 15 (Piggot *et al.*,

Table 2. *Sporulation of strains harbouring plasmids*

Bacteria were induced to sporulate in modified Schaeffer's medium containing 5 µg Cm ml⁻¹ (see Methods); 18 h after the end of exponential growth, a portion of each culture was diluted and plated on nutrient agar (NA), with and without Cm. To determine the extent of sporulation, another portion was heated to 85 °C for 20 min and then diluted and plated. Results shown are for duplicate cultures of each construct.

Bacterial strain and relevant chromosomal genotype	Plasmid	10 ⁻⁷ × Viable cells		10 ⁻⁷ × Heat resistance spores		Percentage sporulation	Percentage Cm ^R spores amongst total spores
		NA	NA-Cm	NA	NA-Cm		
SL1097 (<i>spoVE85 recE4</i>)	pPP7	12.0	7.9	3.3	3.8	28	114
	pPP7	9.5	2.7	2.6	1.5	28	58
	pHV33	5.2	5.9	0*	0*	0	—
	pHV33	7.0	2.1	0*	0*	0	—
	pHV33	13.6	1.0	11.1	0.8	77	7
SL1093 (<i>spo⁺ recE4</i>)	pPP7	14.5	4.2	13.3	1.25	92	9
	pHV33	22.5	15.0	18.7	16.0	83	85
	pHV33	15.9	23.4	16.8	13.8	105	82
	pHV33	15.9	23.4	16.8	13.8	105	82

* No survivors from 0.2 ml undiluted culture.

1985). Clearly *spoVE* does not show the very sharp sensitivity to copy number of *spoOF* where as few as five copies relative to the chromosome inhibit sporulation (Piggot *et al.*, 1985).

Plasmid stability

The structure of plasmid pPP7 was stable in *E. coli* or in *B. subtilis recE4* HsrM⁻ strains such as SL1093 and SL1097 (although it showed segregational instability in *B. subtilis*). Thus with SL1097 as recipient and pPP7 as donor, 100% of Cm^R transformant clones were dark brown. When pPP7 was transformed into Rec⁺ SpoVE strains, however, it was structurally unstable. Thus Cm^R transformants of SL666 (HsrM⁺) and SL1095 (HsrM⁻) had a mixture of colony morphologies with the majority being sectorised for the pigment associated with sporulation; typically, 70% were sectorised, 11% dark brown and 19% very lightly pigmented with no visible evidence of sectoring. On subculture in the presence, or absence, of chloramphenicol the sectorised colonies have a mixture of dark brown, red-brown and poorly pigmented colonies; in the presence of chloramphenicol, sectorised colonies were also obtained. Examination of the colonies with a phase-contrast microscope indicated that the dark-brown colonies were Spo⁺, the red-brown colonies SpoV, and the poorly pigmented colonies Spo⁻ of an undetermined stage that was not V. The colonies grown in the absence of chloramphenicol were Cm^S and presumably had lost the plasmid; they were stable on subculture. This indicates that the chromosome was also subject to change in the presence of the plasmid; the Spo⁺ segregants could be explained by homogenization (Chak *et al.*, 1982), but the Spo⁻ (not stage V) Cm^S segregants could not. The instability was not a function of the particular *spoVE* allele as it was also observed with SL1094 (*spoVE153 rec⁺*). The plasmid used in these experiments was prepared in *E. coli*. It contains sites for *Xho*I (Fig. 2), which is an isoschizomer of the endogenous *B. subtilis* 168 restriction enzyme *Bsu*M (Jentsch, 1983). The instability was not caused by *Bsu*M, however, as it was exhibited by *hsrM* strains that lack *Bsu*M activity. Rather, it appeared to be a consequence of *recE⁺* activity. It was not investigated further.

Isolation of new *spoVE* mutations

The *spoVE* locus was defined by two mutations, *spo-85* and *spo-153* (previously called W5) (Piggot & Coote, 1976). To obtain more chromosomal mutations in the region, pPP7 was mutagenized with hydroxylamine, and used to transform strain MB75 in the presence of an equimolar amount of ϕ 105dlys DNA (Jenkinson & Mandelstam, 1983). The ϕ 105dlys DNA provided a selection for competent MB75, amongst which a proportion might be expected to have plasmid derived *B. subtilis* DNA integrated into the chromosome even though no selection

Table 3. *Localization of spoVE mutations within the cloned region*

Strains with *spoVE* mutations were transformed with plasmids harbouring different portions of the *spoVE* region (see Fig. 2).

Recipient	No. of Spo ⁺ transformants from 0.2 ml transformed culture					
	None	Donor:				
		pPP73	pPP74	pPP75	pPP76	pPP7
SL666 (<i>spoVE85</i>)	18	12	16	14	2240	>4000
MT1 (<i>spoVE1</i>)	0	4	1	692	5	3024
MT2 (<i>spoVE2</i>)	0	660	6	1	3	1300
MT5 (<i>spoVE5</i>)	3	852	2	7	2	2980
MT8 (<i>spoVE8</i>)	0	0	5	784	1	2116

was imposed for plasmid determinants (congression; Nester *et al.*, 1963). Among 10000 Lys⁺ transformants, 31 Spo⁺ mutants were identified in the initial screen; eight of these were found to be stable and transformable to Spo⁺ with pPP7 DNA, suggesting that they were *spoVE* mutants. Four of the eight mutants were asporogenous. The asporogenous mutations were positioned within the cloned region by testing them with plasmids pPP73, 74, 75 and 76, which contained, respectively, the 560, 500, 360 and 220 bp *Hind*III fragments from pPP7 cloned in pJAB1 (Fig. 2). Plasmid pPP76 gave Spo⁺ recombinants with SL666 (Table 3); pPP75 gave Spo⁺ recombinants with MT1 and MT8; and pPP73 gave Spo⁺ recombinants with MT2 and MT5. This indicates that the locus has a minimum size of 560 bp and is likely to be significantly larger than that.

In an attempt to delimit the size of the transcriptional unit, and to see if there is a single unit, we used the integrational plasmid method (Piggot *et al.*, 1984). This employs plasmids that cannot replicate in *B. subtilis*, and have a region of homology with the *B. subtilis* chromosome. When transformants are selected for a plasmid marker that is not in the region of homology, they arise by the plasmid integrating into the region of homology by a Campbell-like mechanism. When the region of homology is entirely within a transcriptional unit, then integration disrupts the transcriptional unit. The method depends upon the correctness of the assumption about the method of plasmid integration, and previous studies have shown it to be valid (Piggot *et al.*, 1984). Unfortunately, it was found not to be valid for the *spoVE* region. Plasmids pPP73, 74 and 75 gave Cm^R transformants of BR151 that were Spo⁺. However, analysis of DNA from the transformants showed that the plasmids had not integrated by a Campbell-like mechanism, so that no conclusions could be drawn above *spoVE* transcription from these experiments.

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