Nucleotide Sequence of the *Bacillus subtilis* Developmental Gene *SpoVE*

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We have determined the nucleotide sequence of a 1159 bp DNA fragment containing the *spoVE* locus of *Bacillus subtilis*. The locus contained a single open reading frame of 293 codons. On the basis of the predicted amino acid sequence, the product of the *spoVE* gene is believed to be a protein with an M_r of 31 539. The amino-terminal portion of the *spoVE* gene was used to construct a translational fusion with the *lacZ'* gene. The hybrid *spoVE-lacZ'* gene was shown to be expressed in *Escherichia coli* and, therefore, it seems reasonable to conclude that the proposed open reading frame for the *spoVE* gene does indeed function *in vivo*.

INTRODUCTION

Bacterial endospore formation, triggered by nutrient limitation, is a primitive type of cellular differentiation. The study of this process in the Gram-positive bacterium *Bacillus subtilis* is particularly amenable to analysis as this organism is probably the most extensively studied of the differentiating prokaryotes.

The completion of the forespore engulfment, resulting in a two-celled structure, marks a significant stage in the process of developmental change in *B. subtilis*. Co-existence of the two genomes in such close proximity inevitably raises a number of questions about their participation in the subsequent biochemical and morphological events. Two major morphological changes that take place after compartmentalization are cortex formation, designated as stage IV, and deposition of the coat layers, designated as stage V. The existence of sporulation mutants which may be cortex⁺ coat⁻ in one instance and cortex⁻ coat⁺ in another, coupled with the results of epistasis experiments with double sporulation mutants, indicates the possibility that more than one dependent sequence of gene expression might be actively involved in the process of spore maturation at later stages (Coote & Mandelstam, 1973; Piggot & Coote, 1976).

There have been extensive studies of cloned sporulation-associated genes (Losick & Youngman, 1984; Piggot, 1985). Most of these are concerned with genes expressed early in sporulation. The *spo* loci involved in the later stages of spore formation are, perhaps, of particular interest because of the complexity of the regulation of the developmental programme with two cell types involved, the mother cell and the forespore. Although most of the late loci are probably expressed only in the mother cell (Lencastre & Piggot, 1979; Dancer & Mandelstam, 1981), only one locus specific to the mother cell, *spoVE*, has been cloned (Yamada *et al.*, 1983; Piggot *et al.*, 1986).

We report here the nucleotide sequence of the *spoVE* locus and the proposed amino acid sequence for its product. We have established that this locus consists of a single gene 879 bp long. The promoter-proximal portion of this gene was used to construct a translational fusion to the *lacZ'* gene. The hybrid *spoVE-lacZ'* gene was expressed in *Escherichia coli*, providing strong evidence that the proposed open reading frame for the *spoVE* locus can be translated *in vivo* and, presumably, is functionally active.

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Table 1. *Escherichia coli* strains

<i>E. coli</i> strain	Genotype	Source/reference
JM103	<i>supE sbcB15 strA thi endA Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^r ZΔM15</i>]	Messing <i>et al.</i> (1981)
JM107	<i>endA1 gyrA96 hsdR17 supE44 relA1 thi, λ⁻ Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^r ZΔM15</i>]	Yanisch-Perron <i>et al.</i> (1985)

METHODS

Bacterial strains, phages and plasmids. The *E. coli* strains used are listed in Table 1.

Plasmid pPP7 is a derivative of the bifunctional vector pHV33, containing a 4.7 kbp chromosomal fragment of *B. subtilis* (Piggot *et al.*, 1986).

Plasmids pUB3 and pUB4 were constructed by subcloning the 1.35 kbp *Xho*I fragment from pPP7 into the *Sal*I site of pUC9 (Vieira & Messing, 1982). The orientation of the insertion was verified by multiple digests with the appropriate restriction enzymes. A physical map of plasmid pUB4 is shown in Fig. 3. Plasmid pUB3 contains the same *Xho*I fragment in the reverse orientation. The construction of plasmid pUB5 is discussed in Results.

Preparation of plasmid DNA. Plasmid DNA was prepared as described by Birnboim & Doly (1979).

Transformation. *E. coli* strains were made competent and transformed by the method of Hanahan (1983). Recombinant M13 or pUC9 vectors were selected as described by Fort & Piggot (1984), except that ampicillin was used at a concentration of 50 µg ml⁻¹ for plasmid selection.

Restriction enzyme analysis and isolation of specific DNA fragments. All restriction enzymes were purchased from BRL, New England Biolabs or Pharmacia. DNA ligase was purchased from Boehringer. Enzymes were used as recommended by the suppliers. Mapping of restriction sites was done by multiple enzyme cutting and analysis of the digestion products by agarose (0.7–1.4%, w/v) gel electrophoresis using Tris/borate buffer (Maniatis *et al.*, 1982). Specific DNA fragments for sub-cloning or sequencing were isolated on horizontal (0.5–1%, w/v) low melting point agarose slabs (BRL) as described by the manufacturers.

DNA sequencing. Specific restriction fragments were sub-cloned into the appropriate M13 (mp10, mp11 or mp19) vector (Messing, 1983) and the nucleotide sequence was determined using the dideoxy chain termination method of Sanger *et al.* (1977). The sequencing kit was purchased from Amersham and was used in accordance with the supplier's recommendations.

RESULTS

The *spoVE* locus has been shown to be contained within a 2.9 kbp *Eco*RI fragment isolated by the 'prophage transformation' method in the temperature phage φ105 (Yamada *et al.*, 1983). The independent isolation of this locus on a plasmid, pPP7 (Piggot *et al.*, 1986), led us to the conclusion that the *spoVE* locus is probably situated on a 1.35 kbp *Xho*I fragment and that its minimum size is about 560 bp. In order to identify the product(s) of the *spoVE* locus and to gain some information concerning the regulation of its expression we have determined the nucleotide sequence of a 1159 bp DNA fragment covering an area where all known *spoVE* mutations were located. A detailed restriction map of the *spoVE* locus and the sequencing strategy are presented in Fig. 1. Plasmids pPP7, pUB3 and pUB4 served as sources of specific DNA fragments for sub-cloning into appropriately cleaved M13 vectors. The areas around all the restriction sites used for sequencing were re-sequenced from a nearby restriction site in order to avoid potential loss of a sequence due to closely spaced restriction sites.

The nucleotide sequence of the 1159 bp chromosomal fragment is shown in Fig. 2. Analysis of the sequence has revealed that there is a single long open reading frame of 293 codons from which a protein (*M*_r 31 539) can be translated. The open reading frame is preceded by a sequence which has a substantial degree of complementarity to the 3' end of the *B. subtilis* 16S rRNA and is, therefore, a potential ribosome binding site (Moran *et al.*, 1982).

With a view to obtaining experimental evidence that we have correctly identified the open reading frame for the *spoVE* gene, we attempted to demonstrate that the N-terminus of this frame can function as a translational start for synthesis of the α-peptide of β-galactosidase (Messing *et al.*, 1977). The hybrid plasmid pUB4 was chosen for subsequent intramolecular alterations aimed at creating a translational in-frame fusion of the *spoVE* and *lacZ'* genes. A

Fig. 1. Physical map of the *B. subtilis* *spoVE* locus. The top line indicates the positions of restriction sites used in sequencing experiments; horizontal arrows represent the sequencing strategy. The position of the large open reading frame (ORF) is indicated by the open box above the restriction map. Av, *Ava*I; Nc, *Nco*I; Nr, *Nru*I; X, *Xho*I; H, *Hind*III. The four *Hind*III fragments are lettered A–C in correspondence to their size.

Fig. 2. Nucleotide and predicted amino acid sequence of the *B. subtilis* *spoVE* locus (only the non-transcribed strand is shown). The sequence was determined on both strands. The strong ribosome-binding site is doubly underlined. The predicted amino acid sequence is shown using standard three letter abbreviations. Broken lines indicate the putative promoter sequences.

1.35 kbp *Xho*I fragment, residing on this plasmid, is in the same translational orientation as the *lacZ'* gene and, according to our sequencing data, does not contain any obvious transcriptional terminators. However, the translational phase of the *spoVE* open reading frame does not coincide with that of the *lacZ'* gene and, indeed, colonies containing pUB4 appear colourless on

Fig. 3. Construction of *spoVE-lacZ'* translational gene fusion. The upper portion of the figure shows the structure of plasmid pUB4. The fragment of the sequence, shown on the right, represents the junction site and demonstrates the frame shift leading to translational failure. The proposed structure of plasmid pUB5 after excision of DNA between *AvaI* and *SmaI* (*AvaI*, *XmaI*) sites of pUB4 is shown in the lower portion of the figure. The sequence across the new junction site is presented on the right. The hatched boxes indicate the *lacZ'* gene. The stippled box represents the area downstream from *spoVE* translational start (TLS2), while the open box indicates the sequence upstream from its ribosome binding site. The direction of translation is from left to right in all cases. Av, *AvaI*; X, *XhoI*; S, *SalI*; Sm, *SmaI*; Nr, *NruI*; TLS1, translational start of the *lacZ'* gene.

medium supplemented with X-gal. We postulated that correlation of the frame shift and reduction in size of the *spoVE* gene portion could lead to at least partial restoration of the α -complementation function of a hybrid peptide (Fig. 3). The experimental approach to test this hypothesis was largely influenced by our discovery that the *AvaI* site within the *spoVE* gene and the *AvaI* (*SmaI*, *XmaI*) site in the multiple covalence site (MCS) area of the vector are in the same translational phase, so that after removal of the DNA fragment between these sites, re-joining of the remaining ends would create an in-frame junction between the *spoVE* and *lacZ'* genes.

Plasmid pUB4 was cut by *AvaI* and the 3.5 kbp fragment was isolated on low melting point agarose. The purified fragment was circularized by T4 DNA ligase and introduced into *E. coli* strain JM103. Altogether, 56-ampicillin resistant transformants appeared on a selective medium containing X-gal and 53 of them showed pale blue colouration. Ten pale blue clones that we analysed contained a plasmid of 3.5 kbp. This new plasmid was designated pUB5. The identity of the remaining *B. subtilis* chromosomal fragment in the plasmid was confirmed by the presence of a unique *NruI* site which is 80 bp upstream from the translational start point of the *spoVE* gene. We verified that this plasmid had the expected structure by subcloning the 0.8 kbp *PstI-EcoRI* fragment of plasmid pUB5 into M13 mp11 and determining the sequence of the junction site between the *spoVE* and *lacZ'* genes.

Position†	Holoenzyme	‘-35’	‘-10’
38-50	$E\sigma^{28}$	CTAAA	CC GATAT‡
		*****	** *** *
		ACTAAAAAACATCGCCTGATT	
49-79	$E\sigma^{43}$	TTGACA	TATAAT
		*** *	*** **
		TTGTTATTGGTCATCATTACGTTATTAT	
125-155	$E\sigma^{37}$	AGNNTT	GGNATTNTT
		* **	*** **
		CGGATTATAAATTGACGACTCATTCTTTT	
181-207	$E\sigma^{32}$	AAATC	TANTGNTTNTA
		****	** * * * *
		GGAATCGGCGTCATTGCTA TGTTTTTT‡	

Fig. 4. Regions showing homology to known *B. subtilis* promoters. Consensus sequences of the *B. subtilis* promoters controlled by minor forms of RNA polymerase were taken from Cowing *et al.* (1985). Nucleotides that match are designated with an asterisk (*). †, The position is given in correspondence to the sequence presented in Fig. 2. ‡, The one residue gap has been introduced in order to make homology more explicit.

DISCUSSION

Two of the *spoV* loci have been cloned so far: *spoVG* (Segall & Losick, 1977) and *spoVA* (Savva & Mandelstam, 1984). A substantial amount of data concerning transcriptional aspects of the *spoVG* locus is now available (Banner *et al.*, 1983). Fort & Errington (1985) have determined the nucleotide sequence of the *spoVA* locus, which is the largest polycistronic sporulation operon yet characterized.

In this paper we present the nucleotide sequence of another stage V locus, *spoVE*, and the predicted amino acid sequence for its product (Fig. 2). The identification of this DNA fragment as the *spoVE* locus is unambiguous since all *spoVE* mutations tested map within this sequence (Piggot *et al.*, 1986). The entire sequence of 1159 bp (Fig. 2) is based on sequencing of individual clones, as indicated in Fig. 1. Computer analysis has revealed one long open reading frame consisting of 293 codons. It starts with an ATG codon at position 240-242. Two nucleotides upstream there is a strong ribosome binding site, GGAGG, for which the free energy of base pairing (ΔG) with the 3' end of the *B. subtilis* 16S rRNA is calculated to be $-14.4 \text{ kcal mol}^{-1}$ ($-60.3 \text{ kJ mol}^{-1}$) using the rules of Tinoco *et al.* (1973). This ΔG value falls into a range published for *Bacillus* (Moran *et al.*, 1982; Fort & Piggot, 1984). An interesting feature of this part of the sequence is the unusual closeness of the ribosome binding site to the translational start point.

Potential transcription initiation signals can be discerned from the determined sequence. Preceding the *spoVE* gene, within 207 bp upstream from the ribosome binding site, are four regions that have a substantial degree of homology to known *B. subtilis* promoters. The positions of these regions are indicated in Fig. 4. The sequences at positions 38-50, 125-155 and 181-207 display quite a close resemblance to promoters recognized by $E\sigma^{28}$, $E\sigma^{37}$ and $E\sigma^{32}$ respectively. All of them appear to belong to the class of promoters utilized by minor forms of RNA polymerase holoenzyme. The sequence at position 49-79 exhibits substantial homology with the consensus sequence of promoters recognized by the major form of holoenzyme $E\sigma^{43}$. Although the exact position(s) of the transcriptional start point(s) will require further investigation, the arrangement of σ^{37} and σ^{32} promoter sequences upstream from the translational start point of the *spoVE* gene is reminiscent of the regulatory region of the *spoVG* locus, which includes the same two types of promoters (Banner *et al.*, 1983).

A great deal of information concerning various aspects of the expression of sporulation genes can be gained by fusing the gene in question to a second gene, whose product can be assayed

Fig. 5. Graphical display of the structural properties predicted for the *spoVE* gene product. (a) The distribution of charged residues along the amino acid sequence. (b) The hydrophobic character of the predicted protein (Kyte & Doolittle, 1982; R. Staden, personal communication). Both profiles were obtained with a segment of seven residues.

quantitatively. We have employed this technique in order to establish whether or not the open reading frame we propose for the *spoVE* locus is translated *in vivo*. Analysis of the *spoVE-lacZ'* structure contained on plasmid pUB4 in terms of translational possibilities provides a convincing explanation for the inability of this plasmid to synthesize a functionally active α -peptide. On the basis of our sequencing data we conclude that disruption of α -peptide synthesis has a translational and not a transcriptional basis. Translation from the original *lacZ'* start point (TLS1) comes to a halt shortly after passing the junction site (*XhoI/SalI*), resulting in a peptide containing 11 amino acids. Translation from the *spoVE* start point (TLS2) will not produce functional α -peptide because of the frame shift (Fig. 3a). In both cases synthesis of a hybrid protein containing α -peptide as a C-terminus appears not to be possible. However, when a portion of the DNA between the *AvaI* site in the *spoVE* gene and the *AvaI* (*SmaI*, *XmaI*) site in the MCS area is removed, the resulting plasmid, pUB5, is apparently able to produce at least partially active hybrid α -peptide, indicated by the appearance of pale blue colouration when plasmid-containing colonies are plated on agar supplemented with X-gal.

We conclude that this excision has created a new in-frame junction between the *spoVE* and *lacZ'* genes (Fig. 3b). Therefore, while the translation from TLS1 remains impeded, translation from TLS2 can now lead to the synthesis of a functionally active hybrid α -peptide whose amino-terminus is substituted for the 118 amino acid stretch of the *spoVE* gene product. It seems reasonable, therefore to conclude that the proposed open reading frame for the *spoVE* gene does indeed function *in vitro*.

The protein product predicted from the nucleotide sequence appears to be quite unusual and shows several features of interest. The ratio of acidic (glutamate, aspartate) or basic (lysine, arginine, histidine) residues is 0.55, indicating a rather basic protein with a calculated isoelectric point of 9.1. This protein also has a high content of hydrophobic residues, which is a

characteristic feature of membrane-bound proteins (Fig. 5). Moreover, it contains several stretches of 18–24 hydrophobic residues flanked by charged residues and this is strongly suggestive of membrane-spanning amino acid sequences (Eisenberg *et al.*, 1984).

The availability of the entire nucleotide sequence of the *spoVE* locus should facilitate the rapid elucidation of those features that are important in the regulation of expression of this gene.

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