Nucleotide Sequence and Complementation Analysis of a Polycistronic Sporulation Operon, *spoVA*, in *Bacillus subtilis*

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We have determined the nucleotide sequence of a 3706 bp stretch of *Bacillus subtilis* chromosomal DNA that complements all known *spoVA* mutations. The sequence contains five consecutive large open reading frames capable of encoding proteins of molecular weights ranging from approximately 15000 to 36000. Analysis using integrational plasmids suggests that the region is likely to be transcribed as a single mRNA. A novel form of complementation analysis, based on derivatives of bacteriophage ϕ 105 carrying the cloned *spoVA* locus, has been used to define four distinct complementation groups among the eight previously characterized *spoVA* mutations. The *spoVA* locus is the largest polycistronic sporulation operon yet characterized.

INTRODUCTION

More than 50 chromosomal loci necessary for normal sporulation and/or germination have been identified by mutagenesis and genetic mapping of the *Bacillus subtilis* chromosome (Piggot & Coote, 1976; Piggot *et al.*, 1981). However, the numbers of genes per locus, and the mechanisms controlling the sequential expression of these genes, have not been elucidated. Several sporulation and germination genes have recently been cloned by a variety of methods (see, for example, Segall & Losick, 1977; Kawamura *et al.*, 1979; Liu *et al.*, 1982; Jenkinson & Mandelstam, 1983; Savva & Mandelstam, 1984; Errington, 1984). However, in most cases it has not been possible to determine whether or not the complete locus was cloned, since each was defined by relatively few *spo* mutations. The nucleotide sequences have been determined for three of these cloned loci: *spoOF* (Shimotsu *et al.*, 1983), *spoOH* (Ramakrishna *et al.*, 1984) and *spoIIA* (Fort & Piggot, 1984). Of these loci, only *spoIIA* was shown to contain more than one gene. Three open reading frames (ORFs), preceded by ribosome binding sites (Shine & Dalgarno, 1974), were identified; they are probably transcribed as a single messenger RNA (Piggot *et al.*, 1984).

A 7 kbp fragment of *B. subtilis* chromosomal DNA, cloned in bacteriophage ϕ 105, was shown to contain both the *spoIIA* locus and a second locus, *spoVA* (Savva & Mandelstam, 1984). The map positions and associated phenotypes of eight mutations in the *spoVA* locus have been determined (Errington & Mandelstam, 1983, 1984). Although the *spoIIA* and *spoVA* loci may be contiguous, they are probably transcribed separately (Piggot *et al.*, 1984). The total map distance between mutations at the extremities of the *spoVA* locus (recombination index, RI, approximately 0.3) was greater than that between the extreme *spoIIA* mutations (RI approximately 0.2) (Errington & Mandelstam, 1984). Thus, it seemed likely that the *spoVA* locus also contained more than one gene.

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Abbreviation: ORF, open reading frame.

We have now used physical and genetic methods to determine the number of genes in the *spoVA* locus, and their organization. We have determined the nucleotide sequence of a 3706 bp long segment of DNA that contains the complete *spoVA* locus. It includes five consecutive ORFs, and we have obtained evidence that the locus is probably transcribed as a single mRNA. Complementation analysis of the eight previously characterized *spoVA* mutations indicates that these lie in at least four different genes, and these genes are tentatively assigned to ORFs in the nucleotide sequence.

METHODS

Bacterial strains, plasmids and phages. These are listed in Table 1, together with their sources.

General methods. Restriction endonucleases and other enzymes were obtained from Amersham, New England Biolabs or BRL, and were used as recommended by the suppliers. Replicative DNA forms of M13mp9, M13mp10 and M13mp11 were obtained from PL Biochemicals (Milwaukee, Wis., USA) or BRL. Preparation of phage ϕ 105 and its DNA, transduction, and other general phage techniques have been described previously (Jenkinson & Mandelstam, 1983). The defective transducing phage, ϕ 105DS1, was provided with wild-type helper phage during transduction (Savva & Mandelstam, 1984). General cloning techniques, preparation of plasmid DNA, ligation and transformation of *Escherichia coli* recipients and Southern hybridizations (Southern, 1975) were performed as described elsewhere (Fort & Piggot, 1984; Errington, 1984).

Cloning in M13 or pUC vectors in *E. coli* is facilitated by a convenient colour indication of the presence of inserted DNA. Insertion of a target DNA in any one of a range of unique restriction endonuclease cleavage sites clustered in a 'polylinker' region results in the loss of β -galactosidase (*lacZ*) activity, which can be detected using the chromogenic indicator, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), as substrate (Messing, 1983).

DNA sequencing. Fragments of *spoVA* DNA, originally from plasmid pPP33 or phage ϕ 105DS1, were sub-cloned in M13mp10 or M13mp11 for sequencing using the chain terminator method of Sanger *et al.* (1977). A combination of exonuclease III and nuclease S1 (Guo *et al.*, 1983) was used to shorten the large (> 1.5 kbp) DNA inserts in two plasmid sub-clones, pPF5 and pPF25, before further sub-cloning in M13. Putative recombinant M13s were analysed by hybridizing the single-stranded phage DNA to DNA from a phage carrying the original

Table 1. *Bacterial strains, bacteriophages and plasmids*

Bacterial strains	Genotype	Source/reference
<i>Escherichia coli</i>		
HB101	F ⁻ <i>hsdS20 supE44 supF58 lacY1</i> or <i>lacIZY76 galK2</i> } <i>galT22 metB1 trpR55</i>	Boyer & Roulland-Dussoix (1969)
JM103	Δ (<i>lac-pro</i>) <i>thi strA supE endA sbcB15 F' traD36</i> } <i>proAB lacI^q ZΔM15</i>	Messing <i>et al.</i> (1981)
JM105	as JM103, but replace <i>supE</i> with <i>hsdR4</i>	Messing (1983)
<i>Bacillus subtilis</i>		
MB75 (Spo ⁺)	<i>metC3 lys-1 tal-1</i>	Laboratory stock
89.7	<i>spoVA89</i>	Errington & Mandelstam (1984)
570	<i>spoVA570</i>	
571	<i>spoVA571</i>	
572	<i>spoVA572</i>	
573	<i>spoVA573</i>	
574	<i>spoVA574</i>	
575	<i>spoVA575</i>	
576	<i>spoVA576</i>	
89.9	<i>spoVA89</i>	Errington & Mandelstam (1984)
570.1	<i>spoVA570</i>	
571.1	<i>spoVA571</i>	
572.1	<i>spoVA572</i>	
573.1	<i>spoVA573</i>	
574.1	<i>spoVA574</i>	
575.1	<i>spoVA575</i>	
576.1	<i>spoVA576</i>	

Table 1. (continued)

Bacterio-phages	Relevant characteristics	Source/reference
M13mp9 } M13mp10 } M13mp11 }	Chain termination sequencing vectors	Messing (1983)
φ105	Wild-type	BGSC*
φ105DS1	<i>spoIIA</i> ⁺ <i>spoVA</i> ⁺	Savva & Mandelstam (1984)
φ105SH3	<i>spoIIA</i> ⁺ <i>spoVA574</i>	S. E. Hartley (unpublished)
φ105J15	<i>spoVA</i> ⁺ †	Errington (1984)
φ105J16	<i>spoIIA</i> ⁺ <i>spoVA570</i> }	This paper
φ105J17	<i>spoIIA</i> ⁺ <i>spoVA576</i> }	
φ105J18	<i>spoIIA</i> ⁺ <i>spoVA571</i> }	
Plasmids		
Stock plasmids	Relevant characteristics	Reference
pUC13	<i>bla</i> 2.7 kbp	Messing (1983)
pSGMU1‡	<i>bla cat</i> 4.7 kbp	Errington (1984)
pPP33†	<i>cat tet</i> 8.9 kbp	Piggot <i>et al.</i> (1984)
Constructed plasmids	Construction§	
pPF5	pUC13, <i>Bam</i> HI, <i>Pst</i> I + 2.1 kbp <i>Bcl</i> I (1), <i>Pst</i> I (2147)	
pPF25	pUC13, <i>Bam</i> HI, <i>Hind</i> III + 2.0 kbp <i>Bgl</i> II (2175), <i>Hind</i> III (unsequenced)	
pSGMU2	pUC13, <i>Nar</i> I + 1.0 kbp <i>Hpa</i> II from pSGMU1	
pSGMU6	pSGMU2, <i>Eco</i> RI, <i>Hind</i> III + (M13mp10) 2.1 kbp <i>Bcl</i> I (2), <i>Pst</i> I (2147)	
pSGMU7	pSGMU2, <i>Eco</i> RI, <i>Hind</i> III + (M13mp9) 1.1 kbp <i>Pvu</i> II (112), <i>Pvu</i> II (1239)	
pSGMU8	pSGMU2, <i>Eco</i> RI, <i>Hind</i> III + (M13mp11) 0.83 kbp <i>Exo</i> III/S1 (1321), <i>Pst</i> I (2148)	
pSGMU9	pSGMU2, <i>Eco</i> RI, <i>Hind</i> III + (M13mp9) 0.96 kbp <i>Pvu</i> II (1227), <i>Pvu</i> II (112)	
pSGMU10	pSGMU2, <i>Eco</i> RI, <i>Hind</i> III + (M13mp10) 0.54 kbp <i>Bgl</i> II (2175), <i>Nar</i> I (2713)	
pSGMU11	pSGMU2, <i>Eco</i> RI, <i>Hind</i> III + (M13mp10) 2.0 kbp <i>Bgl</i> II (2175), <i>Exo</i> III/S1 (unsequenced)	
pSGMU12	pSGMU2, <i>Eco</i> RI, <i>Hind</i> III + (M13mp10) 1.5 kbp <i>Bgl</i> II (2175), <i>Exo</i> III/S1 (unsequenced)	

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† pPP33 and φ105J15 contain the 3.2 kbp *Bgl*II fragment of wild-type *B. subtilis* chromosomal DNA which spans the *spoIIA* and *spoVA* loci.

‡ Previously designated pSG1.

§ The vector and the enzymes used to digest it are given first. After the + symbol the size of the inserted DNA fragment is given, followed by the restriction endonuclease(s) used to generate it. The numbers in parentheses refer to the positions of the cleavage sites in the nucleotide sequence in Fig. 3, except for the first *Pvu*II site in pSGMU9, which is located in the adjacent *spoIIA* locus (see Fort & Piggot, 1984). The DNA inserts in the plasmids derived from pSGMU2 were subcloned from M13mp9, M13mp10 or M13mp11 as indicated. Some of the DNA inserts were derived by shortening of larger fragments using exonuclease III and nuclease S1 (*Exo*III/S1) as described in Methods. The end points of the inserts in plasmids pSGMU11 and pSGMU12 have been determined by sequencing and are 441 bp and 17 bp, respectively, beyond the 3' end of the sequence given in Fig. 3. The former end-point may be within phage-derived sequences (see Results).

full-length insert in reverse orientation. After digestion with nuclease S1, the protected shortened insert was sized on a 1% (w/v) agarose gel (Poncz *et al.*, 1982). Sequencing reactions were performed according to Sanger *et al.* (1980) except that gradient buffer gels and ³⁵S-labelled nucleotides were sometimes used (Biggin *et al.*, 1983).

Complementation analysis. For complementation analysis, partially diploid *B. subtilis* strains were constructed by infecting *spoVA* mutant strains with phage φ105 derivatives carrying cloned portions of the *spoVA* locus. Mutations in the *spoVA* locus were transferred onto the φ105DS1 genome, which carries the complete *spoVA* locus (Savva & Mandelstam, 1984; Errington & Mandelstam, 1984), as follows. A *his*H2 or *lys*-1 auxotrophic strain carrying a *spoVA* mutation was transduced with φ105DS1 by selection for Spo⁺ (see below). The resulting heterozygous Spo⁺ strain was transformed with DNA from a *lys*⁺ or *his*⁺ donor strain, carrying the same *spoVA* mutation. Approximately 3% of the progeny were phenotypically Spo⁻ as a result of recombination between the *spoVA* mutation on the transforming DNA and its wild-type allele on the φ105DS1 prophage (a phenomenon known as congression; Dubnau, 1982).

Fig. 1. Structure of plasmid pSGMU2. This plasmid was constructed by cloning a 1.0 kbp *Hpa*II fragment of DNA containing a selectable chloramphenicol-resistance gene (*cat*) into the unique *Nar*I site of the *E. coli* plasmid vector pUC13 (Messing, 1983). The *Hpa*II fragment was obtained from plasmid pSGMU1 (Errington, 1984) but originated in the staphylococcal plasmid, pC194 (Iordanescu *et al.*, 1978). The thick and thin lines denote DNA from plasmids pSGMU1 and pUC13, respectively. Plasmid pUC13 carries a selectable gene, *bla*, which confers ampicillin-resistance in *E. coli*, and a series of unique restriction endonuclease cleavage sites arranged within the coding region of a part of the β -galactosidase gene (*lacZ'*). In suitable *E. coli* hosts a convenient colour test indicates the presence of cloned DNA fragments within the polylinker region.

For complementation analysis, non-lysogenic strains carrying *spoVA* mutations were grown in Oxoid Penassay Broth to late exponential phase, diluted 10-fold and plated on Schaeffer's agar (Schaeffer *et al.*, 1965) to give a uniform lawn of cells. Phage lysate (20 μ l), made up by mixing equal parts of transducing and 'helper' phage lysates, was spotted on to the lawn and allowed to soak in. The plates were incubated for 24 h at 37 °C, and chloroform vapour was used to select for *Spo*⁺ transductants (Hoch, 1971). If complementation had occurred, *Spo*⁺ lysogenic survivors were apparent within and around the region of lysis caused by the phage infection after further incubation for 2–3 d at 37 °C.

Construction of plasmid pSGMU2. Plasmid pSGMU1 (Errington, 1984), which carries the chloramphenicol-resistance gene originally from plasmid pC194 (Iordanescu *et al.*, 1978), was digested with *Bam*HI, and the 2.0 kbp DNA fragment containing the chloramphenicol-resistance determinant (*cat*) was isolated from a low-melting-temperature agarose gel (0.6%, w/v, BRL) using the method recommended by the suppliers. The fragment was then digested with *Hpa*II and ligated into the unique *Nar*I site of plasmid pUC13. The target fragment contains a site for the *E. coli EcoK* restriction system (nucleotides 1092 to 1105 in the published sequence of plasmid pC194; Horinouchi & Weisblum, 1983), so a restrictionless *E. coli* strain, HB101, was initially used as recipient. Ampicillin-resistant transformants were screened for resistance to chloramphenicol (15 μ g ml⁻¹), and one of the resistant clones was isolated. For further cloning experiments plasmid DNA was prepared from *E. coli* strain JM105, which is also restrictionless but still possesses the *EcoK* modification system. *E. coli* strain JM103 (restriction⁺ and modification⁺) was subsequently used as recipient because of its higher transformation frequency. The structure of plasmid pSGMU2 is shown in Fig. 1.

Analysis of *spoVA* transcriptional units by plasmid integration. Suitable DNA inserts from the M13 clones used in the sequence determination were sub-cloned in pSGMU2 using their common 'polylinker' restriction sites. About 1 μ g plasmid DNA was used to transform *B. subtilis* strain MB75 to chloramphenicol resistance; the selection medium was Oxoid Nutrient Agar containing 5 μ g chloramphenicol ml⁻¹. Chloramphenicol-resistant transformants occur by integration of the plasmid, which cannot replicate autonomously in *B. subtilis*, into the chromosome.

Transformation and sporulation. Methods for transformation of *B. subtilis*, induction of sporulation and measurement of the incidence of heat-resistant spores have been described previously (Errington & Mandelstam, 1983, 1984).

Fig. 2. Sequencing procedure and putative translation products of the *spoVA* locus. The upper part of the figure shows a physical map of the *spoVA* locus including some of the restriction endonuclease cleavage sites, as deduced from the nucleotide sequence. Restriction endonuclease cleavage sites to the right of the *Sal*I site were determined by Southern hybridization experiments (see Results and Piggot *et al.*, 1984). The arrows indicate the direction and extent of individual sequence determinations with the base of each arrow marking the beginning of the sequence (5' end). The solid boxes indicate those areas that were sequenced in both strands; about 80% of the *spoVA* locus was sequenced in both directions. Large ORFs in each of the three possible frames, reading left to right, are shown at the bottom of the figure and are labelled as in Tables 2 and 3. At the top of the figure the solid bars indicate the DNA fragments inserted in phage ϕ 105DS1, plasmid pPP33 and two plasmid sub-clones that were used in the sequencing.

RESULTS

Determination of the sequence

The sources of DNA for sequencing were a 3.3 kbp *Bgl*II fragment contained in plasmid pPP33 (Piggot *et al.*, 1984) and a 7 kbp *Hind*III fragment contained in phage ϕ 105DS1 (Savva & Mandelstam, 1984), as shown in Fig. 2. Plasmids pPF25 and pPF5 were isolated by sub-cloning the 2.0 kbp *Bgl*II–*Hind*III fragment from ϕ 105DS1 and the 2.15 kbp *Bcl*I–*Pst*I fragment from plasmid pPP33, respectively, into plasmid pUC13 (Fig. 2). The inserts in these plasmids were shortened progressively from each end in turn, using exonuclease III and nuclease S1, and were sub-cloned into M13mp10 or M13mp11 as appropriate. The sequencing data obtained were analysed on a computer using the DB system of Staden (1982) to arrange the overlapping sequence determinations into continuous units. In addition, the 1.54 kbp *Pst*I–*Sal*I fragment from ϕ 105DS1 was sequenced from its *Pst*I end to read through the *Bgl*II site, and the 2.29 kbp *Bcl*I–*Hind*III fragment from pPP33 was sequenced from its *Hind*III end (within vector sequences) to read through the *Pst*I site. Other data were obtained by sequencing *Pvu*II fragments sub-cloned from ϕ 105DS1 and a *Nar*I–*Bgl*II fragment from pPF25. The completed sequence of 3706 bp is shown in Fig. 3, and the sequencing procedure in Fig. 2. About 80% of the sequence was determined in both directions; regions sequenced on only one strand were determined from the readings of at least two overlapping M13 clones. The sequence begins at a *Bcl*I site that represents one end of the nucleotide sequence of a DNA segment containing the *spoIIA* locus that was published previously (Fort & Piggot, 1984), and ends at a *Sal*I site.

[illegible]

[illegible]

Analysis using integrational plasmids (see below) suggested that the essential part of the *spoVA* transcriptional unit was contained within the region sequenced. However, Southern hybridization experiments showed that the distal part of the 7 kbp *Hind*III fragment in ϕ 105DS1, i.e. the region beyond the *Sal*I site, contained some phage DNA sequences (data not shown). Presumably the deletion that occurred during the isolation of ϕ 105DS1 (Savva & Mandelstam, 1984) extended into the chromosomal DNA insert. This would also explain the small discrepancy between the restriction maps of the region presented previously (Piggot *et al.*, 1984; Savva & Mandelstam, 1984). To confirm that the sequenced region was composed of chromosomal DNA and not phage sequences, plasmid pSGMU12 DNA (see below) was 32 P-labelled and used as a probe in Southern hybridizations with restriction fragments of chromosomal DNA (*B. subtilis* strain MB75) and phage ϕ 105DI:1t DNA, the vector from which ϕ 105DS1 was derived. The labelled probe hybridized to a *Bgl*II-*Sal*I fragment of chromosomal DNA of the expected size (1.5 kbp) but it did not hybridize to ϕ 105DI:1t DNA (data not shown).

Evidence described below shows that the *spoVA* locus is likely to be transcribed as a single polycistronic mRNA and that the sequence given in Fig. 3 is likely to be the sense strand. Analysis of the sequence using the RUTRANSQ program of Staden (1977) showed six large ORFs covering most of the sequenced region (Figs 2 and 3). Each of the ORFs starts with an ATG codon, which is preceded by a putative ribosome binding site (Shine & Dalgarno, 1974). The free energy values for base-pairing between the putative ribosome binding sites and the 3' end of the 16S rRNA of *B. subtilis* are given in Table 2, the first site being taken from the previously determined sequence of the *spoIIA* region (Fort & Piggot, 1984). The ΔG values calculated (Tinoco *et al.*, 1973) fall within the range -12.0 kcal mol $^{-1}$ (ORF 2) to -22.0 kcal mol $^{-1}$ (ORF5). These are in the same range as those reported for other *B. subtilis* ribosome binding sites (Moran *et al.*, 1982). The first five ORFs are non-overlapping and each terminates

Table 2. *Putative ribosome binding sites preceding spoVA ORFs*

Free energies of base pairing (ΔG) between putative ribosome binding sites (see Fig. 2) and the 3' terminus of the *B. subtilis* 16S ribosomal RNA (McLaughlin *et al.*, 1981) were determined using the rules described by Tinoco *et al.* (1973).

Position*	Sequence†	ΔG (kcal mol ⁻¹)‡	ORF
2060§	GAAAG - A t GTGATC	-14.6	1
608	AAAGGA t c TG	-12.0	2
1043	AAGGAGGT	-20.0	3
1507	AGGAGG a GA	-16.8	4
2532	AGGAGGTG c ATC	-22.0	5
3444	AGAAAGGAG	-18.4	6
16S rRNA	UCUUUCCUCCACUAG-3'-OH		

* Number of the first nucleotide in the given sequence, with reference to Fig. 3.

† Nucleotides complementary to the 3' terminus of the 16S rRNA are shown in upper case.

‡ 1 cal = 4.184 J.

§ Taken from the sequence presented by Fort & Piggot (1984).

Table 3. *Properties of putative spoVA translation products*

	Position*		Number of amino acids	10 ⁻³ × Mol. wt	pI†	Polarity index‡
	Start	Stop				
ORF1	7	606	200	23.1	10.4	40
ORF2	619	1041	141	15.2	10.8	23
ORF3	1056	1505	150	16.1	10.2	32
ORF4	1521	2534	338	36.0	5.4	45
ORF5	2539	3507	323	34.3	5.5	38

* First nucleotide of start codon and last nucleotide before stop codon from Fig. 3.

† Predicted isoelectric point calculated as described by Martin (1964).

‡ Determined using the MWCALC program of Staden & McLachlan (1982).

at or near the ribosome binding site of the following gene. The sixth ORF overlaps the fifth by about 50 bp and extends beyond the region sequenced. The molecular weights and predicted isoelectric points of the putative proteins encoded by ORFs 1 to 5 are shown in Table 3. No ORFs large enough to encode a protein of molecular weight > 5000 were apparent in the presumed antisense strand.

Data from 'Northern' hybridizations using a *spoVA*-specific DNA probe suggests that the *spoVA* mRNA initiates within the region between the *spoIIA* and *spoVA* ORFs (D. Savva, unpublished results). However, a search of the nucleotide sequence of this region revealed no extensive homologies to known *B. subtilis* promoter sequences. The same region contains a possible termination signal for transcription of the *spoIIA* operon (nucleotides 1770 to 1794 in the nucleotide sequence presented by Fort & Piggot, 1984), but no such signals are apparent in the presumptive coding strand of the *spoVA* nucleotide sequence.

Complementation analysis

Since the nucleotide sequence suggested that the *spoVA* locus was polycistronic, it was unlikely that all of the eight previously characterized *spoVA* mutations (Errington & Mandelstam, 1984) would be found to lie in the same gene. To test this prediction, derivatives of phage $\phi 105$ carrying cloned fragments of *spoVA* DNA were used to make partially diploid strains for complementation analysis. $\phi 105DS1$ (Savva & Mandelstam, 1984) carries a 7 kbp DNA fragment that complements all of the known *spoVA* mutations (Errington & Mandelstam, 1984) and also mutations in the adjacent *spoIIA* locus. Phages $\phi 105SH3$, $\phi 105J16$, $\phi 105J17$ and $\phi 105J18$ were constructed by transferring mutations 574, 570, 576 and 571 respectively on to the $\phi 105DS1$ genome as described in Methods. In each case the genotype was verified by showing that the phage was unable to transduce to Spo⁺ strains carrying the same *spoVA* mutation but had retained the ability to transduce to Spo⁺ a strain carrying the *spoIIA69* mutation.

Table 4. Complementation analysis of *spoVA* mutations

A bacterial strain carrying a mutation in the *spoVA* locus was transduced with phage carrying the same or a different *spoVA* mutation. Complementation was indicated by the appearance of numerous survivors (phenotypically Spo⁺) following selection for Spo⁺ transductants. The order of the mutations across the table is as determined in three-factor transformation crosses (Errington & Mandelstam, 1984). +, Transduction of recipient to Spo⁺ by phage; —, no transduction of recipient strain to Spo⁺; (+), Spo⁺ colonies appeared at low frequency (10⁻⁴ per survivor of phage infection; see Results).

Transducing phage	<i>spoVA</i> mutation carried by phage	<i>spoVA</i> mutation carried by recipient:							
		574	575	571	572	570	573	89	576
φ105DS1	None	+	+	+	+	+	+	+	+
φ105SH3	574	—	—	+	—	+	+	+	+
φ105J18	571	+	—	—	—	+	+	+	+
φ105J16	570	+	+	+	—	—	—	+	+
φ105J17	576	+	+	+	+	+	+	—	—
φ105J15	None*	+	+	+	(+)	(+)	—	—	—

* This phage carries a chromosomal DNA insert that comprises only part of the *spoVA* locus (see text).

Strains carrying each of the eight characterized *spoVA* mutations were transduced in turn with each of the phage stocks described above. The results of subsequent Spo⁺ selections are shown in Table 4. Six of the mutations could be sub-divided unambiguously into four complementation groups, denoted by square brackets, as follows: [574]; [571]; [570, 573]; [89, 576]. Two mutations showed anomalous complementation patterns. Mutation 575 was not complemented by φ105SH3 or φ105J18, carrying mutations 574 and 571 respectively, although the latter mutations clearly complement each other. Mutation 572 was complemented by φ105DS1 and φ105J17 but not by any of the other phages.

To confirm that the Spo⁺ phenotype of transductants was due to complementation and not recombination, DNA was extracted from several independent Spo⁺ clones and used to transform strain MB75. In each case about 30% of the Lys⁺ transformants displayed a SpoVA⁻ phenotype (data not shown). This indicates that the Spo⁺ transductants still contained a chromosomal *spoVA* mutation and hence that complementation was responsible for the observed phenotype.

Phage φ105J15 was isolated from a *B. subtilis* genomic library constructed by cloning chromosomal DNA fragments generated by cleavage with *Bgl*III into the unique *Bam*HI site of φ105J9 (Errington, 1984). This phage, which carries a 3.3 kbp insert, was isolated on the basis of complementation of the *spoVA*574 mutation and, unlike φ105DS1 and its derivatives, it is able to plaque and lysogenize normally. Consequently, all of the survivors of infection with this phage are phenotypically Spo⁺. Only the first two groups of mutations were complemented by this phage. Two additional mutations, 570 and 572, though not complemented, appeared to be rescued by recombination; the majority of cells that survived infection remained Spo⁻ but a small number of Spo⁺ survivors (about 10⁻⁴ per survivor) were always present.

Use of integrational plasmids to define the transcriptional unit(s) of the spoVA locus

Since the nucleotide sequence contained six putative proteins but only four complementation groups were defined, it was important to determine whether all six ORFs encoded proteins that were essential for sporulation, and, if so, whether they were transcribed as a single unit. One method of determining the extent of a transcriptional unit is to use integrational plasmids, as described by Piggot *et al.* (1984) for the *spoIIA* operon. The method depends upon the use of a plasmid with two salient properties: firstly, the ability to replicate autonomously in *E. coli* but not in *B. subtilis*; secondly, the expression of an antibiotic resistance gene, in this case Cm^R, that can be selected in *B. subtilis*. Although such a plasmid is incapable of replicating autonomously in *B. subtilis*, Cm^R transformants can be obtained if it contains a fragment of DNA that has homology with the *B. subtilis* chromosome. Such plasmids have been shown to integrate into the recipient chromosome by a Campbell-like mechanism (i.e. by a single crossover event) that

Fig. 4. Analysis of *spoVA* transcriptional units using integrational plasmids. The upper part of the figure shows a physical map of the *spoVA* region (see Fig. 3) and the adjacent *spoIIA* region (Fort & Piggot, 1984). Below, the fragments of DNA subcloned in the integrational plasmid, pSGMU2, are shown with the designations of the derivative plasmids given. Solid lines indicate that the integration of the plasmid into the chromosome of *B. subtilis* strain MB75 (Spo⁺) resulted in a SpoVA phenotype (see Table 5) whereas dotted lines indicate that the phenotype remained Spo⁺.

results in duplication of the homologous DNA insert on each side of the inserted plasmid sequence (Duncan *et al.*, 1978; Haldenwang *et al.*, 1980). The consequences of such an insertion depend upon the end points of the homologous DNA in the original plasmid. If both ends are within a functional transcription unit that is essential for sporulation, insertion will result in a Spo⁻ phenotype. However, if one or both ends of the insert extend beyond the essential part of the functional unit, the Spo⁺ phenotype will be retained.

A versatile integration plasmid, pSGMU2, was constructed as described in Methods. In addition to the properties described above, it carries unique sites for a number of restriction endonucleases, and gives a convenient colour test for the identification of recombinant clones.

Various restriction fragments, some shortened by treatment with exonuclease III and nuclease S1 and used in the nucleotide sequencing, were sub-cloned from phage M13 into plasmid pSGMU2. The size of the DNA insert in each of these pSGMU2 derivatives was checked by restriction analysis and the extent of the homologous insert in each plasmid is shown in Fig. 4. About 1 µg of DNA from each of these plasmids was used to transform *B. subtilis* strain MB75 (Spo⁺) and in each case between 10² and 10³ chloramphenicol-resistant progeny were obtained. The transformants from any one cross were phenotypically homogeneous, being either wild-type or typically SpoVA⁻ as judged by microscopic examination of colonies after 5 d on chloramphenicol-containing agar at 37 °C. The phenotype of each class of transformant was tested more rigorously by inducing sporulation in liquid medium, in the presence of chloramphenicol, and assaying the number of heat-resistant spores at *t*₀. The values for heat-resistant spores (Table 5) were in complete accordance with the results obtained by microscopic examination. The low incidence of spores seen in the transformants recognized as SpoVA⁻ was mainly due to spontaneous reversal of integration, which would remove the block on transcription. In support of this explanation was the observation that >90% of the heat-resistant survivors were found to have become both chloramphenicol-sensitive and Spo⁺ (data not shown). Integration of plasmids pSGMU9, pSGMU11 and pSGMU12 did not produce a SpoVA⁻ phenotype, indicating that at least one end of the homologous inserts in each of these plasmids extends beyond the essential part of the *spoVA* operon.

Southern hybridizations were used to confirm that integration of the pSGMU2 derivatives occurred by the expected Campbell mechanism. Chromosomal DNA, extracted from strain MB75 and a derivative containing plasmid pSGMU10 in the integrated form, was restricted with *Pst*I or *Bgl*II, transferred to nitrocellulose and probed with labelled DNA from plasmid pSGMU10. With both enzymes the hybridization pattern was consistent with the integration of two copies of plasmid pSGMU10, in tandem, by a Campbell-like mechanism (data not shown). The occurrence of tandem duplication of an integrational plasmid has been reported by other authors (Young, 1983; Piggot *et al.*, 1984). We assume, although it has not been established experimentally, that the other pSGMU2 derivatives, including pSGMU9, pSGMU11 and pSGMU12, have integrated by a Campbell-like mechanism.

Table 5. Analysis of *spoVA* transcriptional units using integrational plasmids

DNA (about 1 µg) from derivatives of the integration plasmid, pSGMU2, carrying inserts of *spoVA* DNA (Fig. 4) was used to transform *B. subtilis* strain MB75 (Spo⁺) with selection for chloramphenicol-resistance. Cells from a single isolated chloramphenicol-resistant colony in each cross were induced to sporulate in liquid medium containing chloramphenicol, and the production of heat-resistant spores was determined on non-selective media.

Plasmid	Phenotype*	Heat-resistant spores ml ⁻¹ (t ₉)
pSGMU6	SpoVA ⁻	4.4 × 10 ⁴
pSGMU7	SpoVA ⁻	3.2 × 10 ⁴
pSGMU8	SpoVA ⁻	4.4 × 10 ⁴
pSGMU9	Spo ⁺	4.9 × 10 ⁸
pSGMU10	SpoVA ⁻	1.6 × 10 ⁴
pSGMU11	Spo ⁺	2.6 × 10 ⁸
pSGMU12	Spo ⁺	4.7 × 10 ⁸

* As judged by phase contrast microscopy of sporulating cells.

DISCUSSION

The nucleotide sequence of a 3706 bp DNA segment encoding the *spoVA* locus is presented in Fig. 3. The *Bcl*I site at the beginning of the sequence marks a 6 bp overlap with the 3' end of the nucleotide sequence presented previously for a segment of DNA encoding the *spoIIA* locus (Fort & Piggot, 1984).

The first five ORFs in the *spoVA* locus are all closely coupled; each coding region begins very near to the termination signal of the preceding region. The beginning of the sixth ORF overlaps the fifth by about 50 nucleotides. The reason for this difference is not clear but a similar, although less extensive, overlap has been described for the *B. subtilis* *trpD* and *trpC* genes (Band *et al.*, 1984).

The organization of the *spoVA* ORFs suggest that they constitute a polycistronic operon. We attempted to demonstrate this by making use of integrational plasmids, as described by Piggot *et al.* (1984) (see Results). The results presented in Table 4 and summarized in Fig. 3 suggest that ORFs 1 to 5 form a single transcriptional unit. Insertions beginning before ORF1 (pSGMU9) or after ORF5 (pSGMU11 and pSGMU12) give a Spo⁺ phenotype (as judged by the formation of heat-resistant spores and by microscopic examination) upon integration, and therefore have at least one end outside the essential part of the transcriptional unit. All of the other integrational plasmids conferred a typical SpoVA phenotype upon integration. It remains possible that an increase in gene dosage was responsible for the observed SpoVA phenotype with some integrated plasmids. However, it seems unlikely, in the case of pSGMU10 at least, that two copies of the integrated plasmid would have this effect. Since plasmid pSGMU9 contains a chromosomal DNA insert that overlaps both *spoIIA* and *spoVA* sequences and since it does not disrupt the expression of either upon integration, these operons must be transcribed separately (see also Piggot *et al.*, 1984). The nature of ORF6 may not be clarified until the nucleotide sequence beyond the *Sal*I site is determined unambiguously. This may require cloning a neighbouring chromosomal DNA fragment, perhaps by 'chromosome walking'. It is possible that ORF6 is not actually translated *in vivo*, or that the absence of its product does not confer an obvious Spo⁻ phenotype. For example, it might encode one of the presumably non-essential biochemical marker events that occur at specific stages during sporulation (Mandelstam, 1976), or it could be involved in spore germination or outgrowth. Alternatively, ORF6 may be expressed as an independent overlapping transcriptional unit from a promoter, as yet unidentified, within the *spoVA* operon.

The use of the plasmid pSGMU2 may have some advantages in comparison with the integrational plasmids that have been described previously (for example, pJH101, Ferrari *et al.*, 1983; pJAB1, Piggot *et al.*, 1984). It is particularly versatile because of its polylinker cloning region, with unique sites for a range of restriction endonucleases, and a convenient colour reaction that indicates the presence of inserted DNA. Moreover, the polylinker in pSGMU2 is

identical to those of bacteriophages M13mp10 and M13mp11, which facilitates the rapid subcloning and testing of DNA fragments characterized by sequencing.

Since the *spoVA* locus appears to be transcribed as a single mRNA, the promoter for this transcriptional unit must lie to the left of ORF1 (as shown in Figs 2 and 4) if it is to be compatible with the direction of translation and with the direct complementation of some *spoVA* mutations by phage ϕ 105J15. This phage carries a fragment of DNA that extends from a *Bgl*II site within the *spoIIA* locus through *spoVA* ORFs 1, 2 and 3, to a second *Bgl*II site within ORF4. However, the region between the *spoIIA* and *spoVA* loci (about 300 nucleotides) contains no sequences strongly homologous to any of the known *B. subtilis* promoter sequences.

Although some, at least, of the stage 0 loci are probably monocistronic, several of the later *spo* loci have been shown by complementation analysis and/or sequencing to contain more than one gene: *spoIVC* (Dancer & Mandelstam, 1981) and *spoIVF* (Lamont, 1984) both contain at least two genes; *spoIIA* contains three (Fort & Piggot, 1984). Here we have identified at least five and possibly six genes in the *spoVA* locus.

Six of the eight *spoVA* mutations previously mapped by genetic means and found to have similar phenotypic effects (Errington & Mandelstam, 1984) have been unambiguously divided into at least four different complementation groups (Table 4). In each case mutations in the same complementation group occupy adjacent positions in the genetic map (Errington & Mandelstam, 1984). It is not yet possible to assign the four complementation groups to their respective ORFs with certainty. However, mutations 570 and 573 are probably located in ORF4; although they were not complemented by phage ϕ 105J15, one of them, 570, appeared to be rescued by recombination between the insert in this phage and the chromosome. Presumably 570 lies to the left and 573 to the right of the *Bgl*II site in ORF4. If this assumption is correct, mutations 576 and 89, which map to the right of 570 and 573 (see Table 4) are probably located in ORF5 since ORF6, the only alternative, appears not to be essential for the formation of heat-resistant spores (see above). The other complementation groups cannot yet be assigned to the remaining three ORFs with certainty.

Two of the *spoVA* mutations, 572 and 575 could not be assigned to single complementation groups. Genetic crosses (Errington & Mandelstam, 1984) suggested unambiguous locations for both of these mutations on a linear map. The most likely explanation for the anomalous complementation results (Table 4) is that strains 572 and 575 contain more than one lesion in the *spoVA* region, which is always a possibility after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Hopwood, 1970). Determination of the nucleotide sequence of *spoVA* DNA from these strains should afford an explanation.

Three methods have been described previously for complementation analysis of sporulation genes, but all have inherent drawbacks. Strains carrying the *trpE26* marker contain extensive rearrangements of the *B. subtilis* chromosome, one end of which lies within the *trp* gene cluster (Trowsdale & Anagnostopoulos, 1975). When such strains are transformed or transduced to Trp⁺ the progeny are unstable *trp*⁺/*trpE26* heterogenotes that are partially diploid for a considerable part of the chromosome. This system was used by Karmazyn *et al.* (1972, 1973) and by D. A. Broadbent (cited by Piggot & Coote, 1976) to study the dominance relationships of several sporulation mutations. However, the method is difficult and the analysis of results is time consuming. It does not seem to have been used subsequently to test complementation between pairs of *spo* mutants. The protoplast fusion method cannot be used for mutants blocked before stage III, and though complementation was obtained with all stage IV mutants only one stage V mutant, *spoVF*, was complemented (Dancer & Mandelstam, 1981). Plasmid complementation analysis depends upon the use of Rec⁻ strains, which are not easy to construct, and difficulties can arise from the increase in gene dosage that occurs with multi-copy plasmids (Kawamura *et al.*, 1981; Banner *et al.*, 1983). In contrast, complementation analysis using ϕ 105 is relatively easy, particularly in the light of the recent advances in cloning procedures described for this phage (Errington, 1984). The phage provides a single copy system for constructing partial diploids that is stable even in a Rec⁺ background.

The proteins predicted from the nucleotide sequence of *spoVA* are unusual in several respects. Those encoded by ORFs 1, 2 and 3 have calculated isoelectric points that are rather high (>9.0).

A comparison of the amino acid compositions of these basic proteins with those of the acid-soluble spore-specific proteins (Johnson & Tipper, 1981) showed no obvious similarities. One possible role for such basic proteins would be in binding to DNA for purposes of genetic regulation or for protection during dormancy. The protein product predicted from ORF2 is also very hydrophobic (polarity index 23), and several of the other proteins, particularly that encoded by ORF5, have long tracts (10 or more) of hydrophobic residues, although they are less hydrophobic overall than ORF2. This could indicate that the proteins involved form associations with the membrane or with each other. The similarities of the phenotypic consequences of mutations in different *spoVA* genes would favour the idea that the proteins are functionally related, perhaps as sub-units of a single protein.

The data base provided by the sequencing of the *spoIIA* and *spoVA* loci, together with various vegetative genes such as that for α -amylase (Yang *et al.*, 1983) and the tryptophan operon (Band *et al.*, 1984), enabled a comparison of codon usage in vegetative and sporulation-specific genes to be made, but no notable differences were observed (results not shown). This makes it unlikely that the regulation of sporulation genes is effected at the translational level by the appearance of novel species of transfer RNA.

The identification of at least four essential sporulation genes that are probably coordinately regulated as a single operon leads to a problem in nomenclature that is also applicable to the *spoIVC*, *spoIIA* and *spoIVF* loci. The classical nomenclature of *spo* mutations in terms of genetic loci (Piggot & Coote, 1976) leaves no obvious place for an extra character with which to distinguish different genes within the same locus. To conform with the proposals of Demerec *et al.* (1966), we propose that an extra upper case italicized letter to indicate the gene should be included immediately after the letter defining the particular locus and preceding the allele number of the mutation. Although we identified only four distinct genes in terms of complementation groups, the nucleotide sequencing data suggests that there are at least five structural genes within the *spoVA* operon. We therefore propose that ORFs 1 to 5 correspond to genes that we designate *spoVAA*, *spoVAB*, *spoVAC*, *spoVAD* and *spoVAE*, respectively. Some of the mutations have been assigned unambiguously to their respective ORFs (see above). These we have therefore redesignated as follows: *spoVAD570*, *spoVAD573*, *spoVAE89* and *spoVAE576*. Until such time as the remaining mutations have been unambiguously assigned to a particular gene (ORF), a hyphen will be included within their designations, e.g. *spoVA-574*, again as suggested by Demerec *et al.* (1966).

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