Nucleotide Sequence of the Sporulation Operon, *spoIIIE*, of *Bacillus subtilis*

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A fragment of *Bacillus subtilis* DNA 3490 bp long, capable of complementing *spoIIIE* mutations, was sequenced. The region of the fragment that encodes functions required for sporulation was delimited using integrational plasmids. Sequencing showed that this region contained an operon with two open reading frames together with associated ribosome-binding sites. The deduced translation products would be polypeptides of 518 and 252 amino acid residues. Several sequences resembling promoters recognized by RNA polymerase containing σ^{29} occur in the region preceding the larger open reading frame. Although no transcription-termination signal was identified downstream of the smaller coding region, analysis with integrational plasmids and determination of the size of *spoIIIE* messenger RNA suggest that the locus does not contain a third gene.

INTRODUCTION

Formation of spores by *Bacillus subtilis* requires the sequential expression of about 150 genes (Errington *et al.*, 1985). Cloning of a sporulation gene is the first step in establishing its position in the dependence pattern of sporulation gene expression either by experiments with DNA–RNA hybridization (Savva & Mandelstam, 1985) or by construction of a suitable (e.g. *lacZ*) gene fusion (Errington, 1986). A tentative pattern of the pathway of sporulation gene expression has been described by Turner *et al.* (1986).

Mutations at the *spoIIIE* locus arrest spore development after formation of the prespore protoplast, and block certain later events known to occur only in the spore compartment, e.g. expression of *spoVA* (Errington & Mandelstam, 1986). They have no effect on the synthesis in the mother cell compartment of the enzyme dipicolinic acid synthase, a product of the *spoVF* operon (J. Errington & J. Mandelstam, unpublished results).

In this paper the sequence of a 3490 bp fragment of DNA capable of complementing all known *spoIIIE* mutations is described.

METHODS

Bacterial strains, bacteriophages and plasmids. These are described in Table 1, with details of their sources and construction.

General methods. Replicative forms of M13mp18 and M13mp19 DNA were obtained from Pharmacia. Dephosphorylated *Sma*I-cleaved M13mp10 DNA was from Amersham. Restriction enzymes were used under conditions described by Maniatis *et al.* (1982).

Electrophoresis of DNA was performed through 1% (w/v) agarose (Sigma, type II) with TBE buffer (90 mM-Tris base, 90 mM-boric acid, 2.5 mM- Na_2EDTA , pH 8.3). Gel loading buffer [40% (w/v) sucrose, 0.25% (w/v) bromophenol blue] was added to DNA in TC buffer (10 mM-Tris/HCl, 1 mM-cyclohexanediaminetetra-acetic acid, pH 7.5). DNA was visualized by ethidium bromide fluorescence. Preparative electrophoresis at 4 °C was done with low-melting-point agarose (1%, BRL). DNA was extracted from agarose as described by Savva & Mandelstam (1984).

Ligation mixtures in 10 mM-Tris/HCl (pH 7.5), 50 mM-NaCl, 10 mM- MgCl_2 , 5 mM-DTT, 1 mM-ATP, 1 unit T_4 DNA ligase (BCL), containing approximately 20 ng vector DNA with a 5–10 molar excess of the DNA to be cloned, in a final volume of 10 μl , were incubated overnight at 4 °C.

Table 1. *Bacterial strains, bacteriophages and plasmids*

Bacteria	Genotype	Source/reference
<i>E. coli</i>		
JM103	$\Delta(lac-pro) thi rpsL supE endA sbcB15$ F' <i>traD36 proAB lacI^q ZAM15</i>	Messing <i>et al.</i> (1981)
JM107	$\Delta(lac-pro) thi supE endA relA1 gyrA96 hsdR17$ F' <i>traD36 proAB lacI^q ZAM15</i>	Yanisch-Perron <i>et al.</i> (1985)
GM48	<i>thi thr leu lacY galK galT ara tonA tsx dam dcm supE</i>	Marinus (1973)
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
CU267	<i>leuB16 ilvB2 trpC2</i>	S. A. Zahler*
36	<i>trpC2 spoIIIE36</i>	Piggot (1973)
47	<i>trpC2 spoIIIE47</i>	Piggot (1973)
82	<i>metC3 leu-8 tal-1 spoIIIE82</i>	Hranueli <i>et al.</i> (1974)
Bacteriophages	Relevant characteristics	Reference
M13mp10 } M13mp18 } M13mp19 }	Sequencing vectors	Messing (1983) Norrande <i>et al.</i> (1983) Norrande <i>et al.</i> (1983)
ϕ 105J27 ϕ 105J30 ϕ 105J31	Cloning vector <i>spoIIIE</i> ⁺ <i>spoIIIE</i> ⁺	Jones & Errington (1987) Errington & Jones (1987) Errington & Jones (1987)
Stock plasmids	Relevant characteristics	Reference
pUC18	Ap ^R	Norrande <i>et al.</i> (1983)
pSGMU2	Ap ^R Cm ^R integrational plasmid	Fort & Errington (1985)
Constructed plasmids		Construction†
pSGMU203		pUC18 <i>SmaI XbaI</i> + 3.5 kbp <i>SmaI XbaI</i> ϕ 105J30
pSGMU204		pSGMU2 <i>SmaI</i> + 1.1 kbp <i>PvuII</i> 103, 1182
pSGMU205		pSGMU2 <i>SmaI</i> + 0.65 kbp <i>HincII</i> 2502, 3140
pSGMU208		pSGMU2 <i>HindIII</i> + 0.90 kbp <i>HindIII</i> 2120, 3038
pSGMU209		pSGMU2 <i>HindIII</i> + 0.48 kbp <i>HindIII</i> 3038, 3490‡
pSGMU210		pSGMU2 <i>SstI</i> + 0.70 kbp <i>SstI</i> 1‡, 688
pSGMU211		pSGMU2 <i>SstI</i> + 0.86 kbp <i>SstI</i> 1134, 1995
pSGMU212		pSGMU2 <i>SstI</i> + 1.40 kbp <i>SstI</i> 1995, 3422

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† Shows enzymes used in the preparation of vector DNA followed by the size of the cloned fragment, the enzymes used to generate the fragment and its origin. Plasmid pSGMU203 was the source of fragments for the other seven plasmids. Positions of the ends of each fragment from the sequence shown in Fig. 3 are given.

‡ Two constructions involved the use of restriction sites in the polylinker region of pSGMU203 (derived from pUC18) which are adjacent to termini of the 3.5 kbp fragment. The numbers represent the end of the sequenced region adjacent to the sites in the polylinker, not the actual position in Fig. 3.

Fragments of DNA generated with *HaeII* were ligated to *SmaI*-cleaved vectors after removal of protruding 3' ends with DNA polymerase I Klenow fragment. DNA (approximately 100 ng) was incubated with Klenow fragment (1 unit) in 10 mM-Tris/HCl (pH 8.0), 5 mM-MgCl₂ for 30 min at 37 °C. Deoxynucleotide triphosphates (100 µM each) were added and the incubation continued for a further 1 h at room temperature.

DNA-DNA hybridizations were done as described by Southern (1975). RNA for 'Northern' hybridizations was prepared according to Savva & Mandelstam (1985). After electrophoresis through 1.5% (w/v) agarose in the presence of formaldehyde, as described by Maniatis *et al.* (1982), RNA was transferred and hybridized according to Savva & Mandelstam (1985).

Escherichia coli techniques. Strains of *E. coli* were made competent as described by Fort & Piggot (1984). Transformants of strains JM103 and JM107 were screened for plasmids containing DNA inserts by their inability to hydrolyse 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (Messing, 1983).

Plasmids were prepared by the alkaline lysis method of Birnboim & Doly (1979) followed by CsCl/ethidium bromide density-gradient centrifugation (Lovett & Keggins, 1979). Replicative-form DNA was prepared from cells infected with M13 vectors and grown as described in the Amersham M13 Cloning and Sequencing Handbook. DNA was extracted and purified by the CsCl/ethidium bromide centrifugation method used for plasmid preparation. Typically this was done to 'turn-around' cloned fragments of DNA to permit sequencing from the other end.

DNA sequencing. This was done by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) as described in the Amersham M13 Cloning and Sequencing Handbook. Sequencing primer was annealed to template DNA by heating the annealing mixture at 100 °C for 3 min then allowing it at least 15 min to cool to room temperature (New England Biolabs Sequencing Manual). Sequencing reactions were done with α -[³⁵S]dATP (Amersham), at 37 °C. All nucleotides were from BCL.

The C-test described by Messing (1983) was used to check certain M13 clones for the correct insert and its orientation. Samples (20 μ l) of phage supernatant to be tested and of the phage which contained the appropriate complementary insert were mixed with 4 μ l 100 mM- Na_2EDTA (pH 8.0), containing 1% (w/v) SDS, 50% (w/v) glycerol and 0.1% bromophenol blue, and incubated for 1 h at 65 °C prior to electrophoresis.

***B. subtilis* techniques.** *B. subtilis* was made competent for transformation by the method of Anagnostopoulos & Spizizen (1961) as modified by Jenkinson (1983). Chloramphenicol was added at 5 $\mu\text{g ml}^{-1}$ to nutrient agar for selection of chloramphenicol-resistant transformants. Selection for Spo^+ transformants and transductants was on Schaeffer's sporulation agar (Schaeffer *et al.*, 1965) with chloroform treatment (Hoch, 1971) as described by Errington & Jones (1987).

Sporulation was induced by the resuspension method of Sterlini & Mandelstam (1969) and times after induction are given as t_0 , t_1 , t_2 , etc. The incubation temperature was 37 °C. Alkaline phosphatase activity was assayed according to Errington & Mandelstam (1983). Heat-resistance was determined by heating samples of resuspended cultures, taken at t_0 and diluted tenfold with resuspension medium, at 80 °C for 15 min. The number of survivors was determined by plating dilutions on nutrient agar (Oxoid).

RESULTS

Isolation, characterization and complementation efficiency of recombinant bacteriophages ϕ 105J30 and ϕ 105J31

Two derivatives of ϕ 105J27 which transduced a *spoIIIE* mutant to Spo^+ were isolated by Errington & Jones (1987) from a genomic library formed by cloning size-fractionated (2–4 kbp) *Mbo*I partially digested *B. subtilis* 168 DNA into the unique *Bam*HI cloning site of ϕ 105J27 (see Fig. 1*b*). The ability of ϕ 105J30 and ϕ 105J31 to complement the *spoIIIE36* mutation was demonstrated by subsequently inducing the phage in Spo^+ transductants and repeating the transduction of strains carrying *spoIIIE36* to Spo^+ . Both phages were also able to transduce *spoIIIE47* and *spoIIIE82* mutants to Spo^+ as shown by the cross-streaking test on Schaeffer sporulation agar (Errington & Jones, 1987).

The efficiency of sporulation was compared in the three *spoIIIE* mutants and their ϕ 105J30 lysogen derivatives. Production of alkaline phosphatase was monitored at t_4 to ensure that sporulation was proceeding normally at least up to stage II and heat resistance was measured at t_0 (see Methods). Since ϕ 105J27 and its derivatives are temperature-inducible (Jones & Errington, 1987) incubation at 37 °C, instead of 30 °C, could reduce viability by causing induction of the phage. Accordingly, a Spo^+ strain, CU267, and a CU267 ϕ 105J27 lysogen were also induced to sporulate by resuspension at 37 °C. Sporulation in all the ϕ 105 lysogens was normal as adjudged by the numbers of heat-resistant colony-forming units (Table 2). The temperature-inducible nature of ϕ 105J27, and presumably of its derivatives, had no noticeable effect on the yield of spores.

Table 2. *Complementation of spoIIIE mutations by phage ϕ 105J30*

Strains were lysogenized by streaking on agar spread with phage ϕ 105J30 (Errington & Jones, 1987). Formation of heat-resistant spores was determined at t_0 after resuspension in sporulation medium at 37 °C (Sterlini & Mandelstam, 1969).

Strain	Heat-resistant spores ml^{-1}	
	Non-lysogen	Lysogen*
CU267 (wild-type)	2.1×10^8	2.5×10^8
36	$< 10^3$	5.6×10^8
47	1×10^4	2×10^8
82	1.4×10^4	1×10^8

* CU267 was lysogenized with ϕ 105J27.

Fig. 1. Restriction endonuclease analysis of bacteriophages $\phi 105J30$ and $\phi 105J31$. (a) DNA from $\phi 105J27$ and the two *spoIIIE* bacteriophages $\phi 105J30$ and $\phi 105J31$ derived from it were digested with *SmaI* and *XbaI* and subjected to electrophoresis in agarose gels. Lanes: 1, $\phi 105J27$; 2, $\phi 105J30$; 3, $\phi 105J31$. Figures for the sizes of the *SmaI* fragments (kbp) are taken from Jones & Errington (1987). (b) Restriction map of $\phi 105J27$ showing *SmaI* sites and the polylinker section containing the *BamHI* cloning site into which DNA was ligated forming $\phi 105J30$ and $\phi 105J31$. (The inserts in $\phi 105J30$ and $\phi 105J31$ are not drawn to the same scale as the map of $\phi 105J27$).

Preliminary restriction mapping revealed that $\phi 105J30$ and $\phi 105J31$ contained fragments of *B. subtilis* DNA of 3.5 kbp and 3.3 kbp respectively. Fig. 1(a) shows the fragments of DNA generated by digestion of DNA from $\phi 105J27$, $\phi 105J30$ and $\phi 105J31$ with the restriction endonucleases *SmaI* and *XbaI*. The pattern obtained with $\phi 105J27$ DNA (Fig. 1a, lane 1) was essentially identical to that obtained when *SmaI* was used alone, since the unique *XbaI* site in the polylinker region is adjacent to a *SmaI* site (see Fig. 1b). Any DNA cloned into the unique *BamHI* site of $\phi 105J27$ is thus immediately flanked by sites for *SmaI* and *XbaI*. Provided that neither enzyme cuts within the insert, as is the case with the inserts of $\phi 105J30$ and $\phi 105J31$, double digestion with both enzymes provides a convenient means of excising the cloned fragments precisely. The cloned fragments from $\phi 105J30$ and $\phi 105J31$ are labelled X (3.5 kbp) and Y (3.3 kbp) respectively, in Fig. 1(a). All three lanes in Fig. 1(a) show an extra band above the bands at 9.7 kbp. This results from annealing of 9.7 and 6.2 kbp fragments mediated by the phage cohesive ends.

The use of restriction enzymes, such as *HindIII* or *SstI*, that cleave both within the insert and the vector confirmed that the inserts differed only slightly in length. The difference of 0.2 kbp presumably results from the absence of one *MboI* fragment from the $\phi 105J31$ insert (this was deduced from the sequence). Both inserts are in the same orientation, the difference between them being at the *SmaI* end.

Construction of plasmid pSGMU203

To construct a detailed restriction map of the fragments which complement all three *spoIIIE* mutations, the 3.5 kbp *SmaI*–*XbaI* fragment from $\phi 105J30$ was subcloned into the *E. coli* plasmid pUC18 (Norlander *et al.*, 1983). Phage DNA digested with *SmaI* and *XbaI* was electrophoresed through low-melting-point agarose. The appropriate fragment was isolated and ligated with similarly digested pUC18. To avoid any problems that might be caused by the *E. coli* K12 restriction system, strain JM107 was used as the recipient in the initial transformation. Transfer to restriction-proficient strains JM103 and GM48 was then possible. The latter strain is *Dam*[−] and *Dcm*[−]; thus plasmid DNA isolated from this strain can be digested by restriction endonucleases (such as *BclI*) inhibited by methylation at their recognition sites. The resultant

Fig. 2. Partial restriction map of the 3.5 kbp *spoIIIE* fragment from $\phi 105J30$. The map was derived from digests of pSGMU203 DNA. The sites shown are mostly those used to construct M13 sequencing vector clones. The arrows indicate that start and extent of the sequences obtained from these clones. The left-hand end of the map corresponds to the *SmaI* site of $\phi 105J30$.

plasmid, named pSGMU203, was used to derive the restriction map shown in Fig. 2. Sites shown are principally those that were used for DNA sequencing. Agarose gel electrophoresis failed to detect the very small fragments generated by *HaeII*, *HindIII* and *RsaI* at positions 2240 to 2276, 2100 to 2120 and 2689 to 2712 respectively (as numbered in the nucleotide sequence shown in Fig. 3). The presence of the extra sites for these enzymes was deduced from the nucleotide sequence.

Plasmid pSGMU203 DNA was also tested for the ability to transform *spoIIIE* strains to Spo^+ . More than 1×10^4 Spo^+ transformants per μg DNA were obtained with strains 47 and 82. The other strain which was used in the initial cloning in $\phi 105J27$, *spoIIIE36*, gave only about 10^3 transformants per μg DNA. This difference could be due to the mutation being very near to the end of the cloned fragment with a corresponding reduction in the incidence of recombination.

Fragments from pSGMU203 were subcloned further into the integrational plasmid pSGMU2 (Fort & Errington, 1985) primarily for use in determining the extent of the *spoIIIE* functional unit within the cloned fragment, but also to provide a more convenient source of fragments for determination of the nucleotide sequence.

To confirm that the 3.5 kbp fragment cloned in pSGMU203 had not resulted from ligation of non-adjacent *MboI* fragments of *B. subtilis* chromosomal DNA, a Southern blotting experiment (Southern, 1975) was performed as follows. Chromosomal DNA from *B. subtilis* was digested with *SstI* or *PvuII*, subjected to electrophoresis, blotted and then probed with a nick-translated sample of pSGMU203. Hybridization occurred to a *PvuII* fragment of 1.1 kbp and to *SstI* fragments of 1.43, 0.86 and 0.45 kbp, as expected from the restriction map of pSGMU203 (data not shown). This confirms that the segment is a part of the *B. subtilis* chromosome that has not suffered rearrangement.

Determination of nucleotide sequence

Restriction fragments of the 3.5 kbp *SmaI*–*XbaI* fragment were derived from plasmid pSGMU203 or pSGMU2 derivatives (see Table 1) and subcloned into M13 chain termination sequencing vectors. The origins of the segments are shown in Fig. 2 with the maximum extent of sequence derived from each clone. The complete nucleotide sequence is shown in Fig. 3. Both strands of the fragment were sequenced to the right of the *SstI* site at position 688. The remaining section of the fragment was sequenced from at least two independent clones unless both strands had been sequenced.

It was necessary to substitute dITP for dGTP to enable sequencing of both strands around the region containing two *HaeII* sites at positions 2240 and 2276 to eliminate so-called compressions from sequencing gels.

Autoradiograms were read and recorded using a sonic digitizer (Staden, 1984a). The complete sequence was compiled using the DBUTIL program of Staden (1982). Analysis of the nucleotide

E G D Y E T V S P L I H S E P I I S S F S D R N E E E S P V I E K R A E P V S
 AAGGGGATTAAGAACAGATCGCCTCTTATTCATTCAGAGCGGATATCTCAAGCTTTCTTCAGATTAAGAAAGCGCCGCAACCTGTGT
 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920

K P L Q D I Q P E T G D Q E T V S A P P M T F T E L E N K D Y E M P S L D L L A
 CGAAGCCGCTTCAGGACATCCAAACCGGACAGGTGATCAGAACTGTTCCTCTCTATGACCTTTACAGAGCTCGAAATAGGATTAGAGATCGCGTCACTGATTGCTGG
 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040

D P K H T G Q Q A D K K N I Y E N A R K L E R T F Q S F G V K A K V T Q V H L G
 CAGATCCGAGCATACCGCCAGCAGCGCTGATAAAGAAATATTTATGAAATTCGGAAGAGCTTGAACGCACATTCCAAAGCTTTGGCGTAAGCGGAAGTCACACAGGTTCACTTCG
 2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160

P A V T K Y E V Y P D V G V K V S K I V N L S D D L A L A K D I R I E A P
 GACCGCGTAAACAAATATGAGTATCTGATGTCGAGTGAAGTTAGCAAAATGTCAATTAAGCGATGATTAGCGCTTTCGCTGCGAAGGATATCAGATCGAAGCGC
 2170 2180 2190 2200 2210 2220 2230 2240 2250 2260 2270 2280

I P G K S A I G I E V P N A E V A M V S L K E V L E S K L N D R P D A N V L I G
 CTATACAGGCAATCAGCAATCGGAATGGAAGTTCCAAAGCAGAGTGGCAATGCTTCGCTGAGAAAGTCTAAATGGAATGACAGACCGGATGCGAACGTGTTAATTG
 2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390 2400

L G R N I S G E A V L A E L N K M P H L L V A G A T G S G K S V C V N G I I T S
 GATCGCGCATATTCAGTGAAGCTGATGGCTGAGTTAAACAGATCCCGCTTTTGGTCCAGGCGCAACCGGTAGCGGAAAAGTGTCTGTGCAACGGCATTTATACAA
 2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520

I L M R A K P H E V K M M I D P K M V E L N V N G F R I C L L L L *
 GTATTTTAAATCGGCGAAGCAGCATGAAATGAAATGATGATCGATCCTTAAATAGTAGAGCTAAATCTACACGATTCGCGATTTGCTTCCTCTGTTGTAAACCGATCCGAA
 2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640

AAAGCGTCGAGCTTGA^{spoIIIEB}AAAGTTGTCAATGAAATGAGCGCGTGAACGCTTTCTCATCGGTACAGAAATTTGAAGGATACAAATGATATATAAACGCGCAACAA
 2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760

E E G A K Q P E L P Y I V I V D E L A D L M M V A S D V E D S I T R L S Q M
 TGAAGAAGAGCAACAGCCAGAGCTGCCTTATTTGTCATTGGAAGAGTACCGGATCTCATGCTGCTTCATCTGATGGAAGATTCGAGATTCGAGATTTGCCCAAT
 2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880

A R A G I H L I I A T Q R P S V D V I T G V I K A N I P S R I A F S V S Q T
 GCGCGGCTCGCGATCCATCTTATTATTCGACACAGCGCCATCGGTGATGTTATCACAGGGTATCAAGCGAATTCGCTCAGCAATCGGTTCCAGGTATCTTCACAGAC
 2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000

D S R T I L D M G A E K L L G R G D M L F L P V G A N K P V R V Q G A F L S D
 GGATTCAGGACCATTTGACATGGTGGCTGAGAAGCTTCTGGCGCGGATATCTGTTTACCTGTAGGCTTAACAACTGCTCGGTGCGAGGAGCATTTTGTGCGA
 3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120

D E V E K V D H V I T Q Q K A Q Y Q E M I P E E T T E H S E V T D E L Y D
 TGCAAGTGGAGAGTCTCGACCATGTAATTAATCAGCAGAAAGCAATATCTCGAGGAGCAAGCAATCTATCCGAGGTACTGTAATGAACTTATGA
 3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240

E A V E L I V G M Q T A S V S M L Q R F R I G Y T R A A R L I D A M E E R G V
 TGAAGTGTGAATTAATGTCGCAATGACAGCGCATCTGTTTCAATGCTGCAAGAAGATTCAGAAATTTGCTATACGAGAGCGCGCTGATTCAGCAATGAGGAGACCGCGT
 3250 3260 3270 3280 3290 3300 3310 3320 3330 3340 3350 3360

V G P Y E G S K P R E V L L S K E K Y D E L S S *
 TGTGCGACGTGAGCGCAACCTGAGGAATTTATATCAAGAGAAATATGATGAGCTCTCTTCTTAATGAGGAGGATTCGCGCTTCTATAGTTGTCATATGATATTATG
 3370 3380 3390 3400 3410 3420 3430 3440 3450 3460 3470 3480

TAAACTGATC
 3490

Fig. 3. Nucleotide sequence of the *spoIIIE* region. Putative protein products are indicated using the standard single-letter code for amino acids. Ribosome-binding sites are indicated by unbroken underlining. Promoter sequences recognized by σ^{54} -containing RNA polymerase are indicated by broken underlining. Nucleotide number 1 corresponds to the left-hand end of the restriction map shown in Fig. 2 (labelled 0). The last digit of numbers below the sequence is aligned with the nucleotide of that number.

Table 3. *Potential ribosome-binding sites and properties of the putative translation products of the spoIIIE operon*

	Ribosome-binding sites*		$\Delta G^\dagger$ (kcal mol ⁻¹)		
<i>spoIIIEA</i>	a G t A A G G t G a T G A g t g t g g c a		-10.8		
<i>spoIIIEB</i>	a a A A G G t t G T c A a t g a a a t g		-9.8		
16S rRNA‡	3' U C U U U C C U C C A C U A G				
	Translation products				
	Nucleotide position§		No. of amino acids	<i>M_r</i>	pI
	Start	Stop			
<i>spoIIIEA</i>	1074	2627	518	57291	8.9
<i>spoIIIEB</i>	2678	3433	252	28143	4.5

* Ribosome-binding sites begin at positions 1062 and 2662. Nucleotides complementary to the 3' terminus of 16S rRNA are shown in upper case.

† Free energy of base pairing calculated by the rules of Tinoco *et al.* (1973); 1 kcal = 4.184 kJ.

‡ The sequence of the 3' terminus of *B. subtilis* rRNA was taken from McLaughlin *et al.* (1981).

§ Position of first nucleotide of start codon and last nucleotide before stop codon in Fig. 3.

|| Estimated according to Martin (1964).

sequence with the ANALYSEQ software package of Staden (1984*b*) revealed three open reading frames, all of which were on the same strand. This would account for over 80% of the full coding potential of the fragment. Each open reading frame occurs in a different reading phase. No major open reading frames were found on the other strand. The three open reading frames are at positions 1 to 732, 1056 to 2627 and 2576 to 3433.

The first open reading frame, ORF X in Fig. 3, represents a fragment of a gene whose function is unknown and which is not concerned in sporulation as demonstrated by analysis with integrational plasmids (see below). The remaining two open reading frames were shown to be within regions of the sequenced fragment concerned with spore formation.

Potential ribosome-binding sites were located to define the possible protein products encoded in the open reading frames. Translation products encoded by the regions in the sequence from positions 1074 to 2627 and 2678 to 3433 start with known initiation codons preceded by regions complementary to the 3' end of 16S ribosomal RNA permitting a ribosome-binding interaction (Shine & Dalgarno, 1974). In accord with the convention of Fort & Errington (1985) the two sporulation genes coded between 1074 to 2627 and 2678 to 3433 were named *spoIIIEA* and *spoIIIEB* respectively. Table 3 shows the ribosome-binding sites and the properties of the translation products. The method of Tinoco *et al.* (1973) was used to estimate the free energies of the Shine-Dalgarno interactions. Both interactions were considered to be rather weak in view of the accepted notion that the mRNA-16S rRNA interaction in *B. subtilis* is more stringent than that in *E. coli*, with an average free energy value of -17 kcal (71.1 kJ) mol⁻¹ as compared with -11 kcal (46.0 kJ) mol⁻¹ (Murray & Rabinowitz, 1982; Gold *et al.*, 1981). However, the values obtained are acceptable, as Wang & Doi (1986) have demonstrated the utilization of a ribosome-binding site by *B. subtilis* with an estimated free energy for the Shine-Dalgarno interaction of -9.2 kcal (38.5 kJ) mol⁻¹.

Transcription-initiation signals are summarized in Table 4. Three regions resembling σ^{29} promoter sequences can be located in the region between the end of ORF X and the start of the putative *spoIIIEA* coding region. While there is good agreement with the consensus σ^{29} promoter sequence in terms of the most highly conserved bases (Cowing *et al.*, 1985), there are discrepancies in the spacing between the -35 and -10 regions. Similarities to other promoter sequences were nowhere near as marked. For example, a σ^{32} -35 region could be seen at position 868 and a σ^{43} -19 region at 977. However, no corresponding -10 and -35 regions could be seen.

Table 4. Potential transcription-initiation signals in the *spoIIIE* locus

The consensus sequence for σ^{29} is taken from Cowing *et al.* (1985) and the most highly conserved bases are underlined.

Nucleotide position*	-35	Spacer	-10
865	TTCAAA	20	CATTTT
1010	TGTAAA	13	CATTAT
1027	TTCATT	13	CATTTT
σ^{29} consensus	<u>TTN</u> AAA	14-17	<u>CAT</u> ATT

* Position in Fig. 3.

Table 5. Analysis of the *spoIIIE* region using integrational plasmids

B. subtilis strain 168 was transformed with each integrational plasmid and chloramphenicol-resistant transformants were selected. A transformant resulting from each plasmid was induced to sporulate by resuspension (Sterlini & Mandelstam, 1969). Sporulation was monitored at t_4 for alkaline phosphatase. The formation of heat-resistant spores at t_9 was determined. Survivors were plated on nutrient agar containing chloramphenicol ($5 \mu\text{g ml}^{-1}$).

Integrational plasmid	Transformants per μg plasmid DNA	Phenotype*	Heat-resistant spores ml^{-1}
pSGMU204	1.1×10^4	Spo ⁺	2.1×10^8
pSGMU205	0.65×10^4	Spo ⁻	1×10^4
pSGMU208	0.40×10^3	Spo ⁻	$< 1 \times 10^3$
pSGMU209	0.60×10^3	Spo ⁺	3.4×10^8
pSGMU210	2.2×10^3	Spo ⁺	1×10^8
pSGMU211	1.5×10^3	Spo ⁻	1×10^5
pSGMU212	0.85×10^3	Spo ⁻	2×10^5

* Examination of colonies on Schaeffer's agar containing chloramphenicol for the presence of phase-bright spores.

No obvious stem-loop structure resembling a potential transcription-termination signal can be found in the short region downstream of the *spoIIIEB* coding region. Thus, the existence of a third gene in the operon could not be precluded on sequence data alone.

The region of the complete sequence labelled ORF X in Fig. 3 represents the C-terminal fragment of an ORF of unknown function. Again, no transcription-termination sequence is obvious in the region between the end of ORF X and that start of *spoIIIEA*.

Delimitation of the *spoIIIE* transcriptional unit using integrational plasmids

The restriction fragments cloned into the integrational plasmid pSGMU2 span the full length of the 3.5 kbp fragment. Transformation of competent wild-type *B. subtilis* with a plasmid, incapable of replicating in this host, containing homologous DNA and a selectable antibiotic resistance can result in a single recombination event similar to that when bacteriophage λ integrates into the *E. coli* chromosome. When the whole of the region of homology is contained within a transcription unit, disruption of that unit must occur, giving rise to a mutant phenotype. If only part of the region of homology is within the functional unit, disruption with change in phenotype does not take place (Piggot *et al.*, 1984).

Plasmids used in such an analysis of the *spoIIIE* locus are described in Table 1. The position of the cloned fragments relative to the open reading frames is shown in Fig. 4. The Spo phenotypes of chloramphenicol-resistant transformants were characterized by (a) their appearance when patched onto Schaeffer's sporulation agar, (b) examination under the phase-contrast microscope, and (c) the incidence of heat-resistant spores in cultures induced to sporulate by the resuspension method of Sterlini & Mandelstam (1969). The results are given in Table 5.

Fig. 4. Relationship between open reading frames in the *spoIIIE* region and the DNA fragments used in the integrational plasmid analysis. Translation start sites are indicated by the small vertical lines.

Plasmids pSGMU205, pSGMU208, pSGMU211 and pSGMU212 disrupted the *spoIIIE* locus so that the region covered by the inserts in these plasmids must be within the *spoIIIE* functional unit. However, plasmids pSGMU204 and pSGMU209 gave sporulation-positive chloramphenicol-resistant transformants. Both plasmids overlap the ends of the segment in the *spoIIIE* locus defined by pSGMU211 and pSGMU212. The opposite ends of the *spoIIIE* functional unit therefore appear to be in the fragments cloned in pSGMU204 and pSGMU209, although the latter, as already mentioned, does not contain any obvious transcription-termination signal after the coding region of the *spoIIIEB* gene.

RNA extracted from sporulating *B. subtilis* at various times after the induction of sporulation (t_0 – t_3) was analysed by 'Northern' hybridization with nick-translated pSGMU212 DNA as a probe. The probe contains a 1.4 kbp *Sst*I fragment which extends from position 1995 to 3422 of the sequence. An RNA species of about 2.5 kb was detected at t_3 (data not shown). If this represents transcripts initiated from the putative promoters listed in Table 4, it suggests that a transcription termination signal exists near the end of the region which was sequenced.

DISCUSSION

A 3.5 kbp fragment of *B. subtilis* DNA capable of complementing the three known *spoIIIE* mutations has been sequenced. The *spoIIIE* locus was shown to consist of a dicistronic operon, thus adding another example to the list of sporulation loci which are known to contain more than one gene.

The deduced translation products of the two *spoIIIE* genes show several interesting characteristics. *spoIIIEA* codes for a large polypeptide of 518 amino acids (M_r 57 300). There is a relative lack of acidic residues and quite a large number of hydrophobic residues. Most of the hydrophobic residues lie in the amino-terminal part of the protein. However, the extreme amino-terminal end is quite highly charged. The smaller *spoIIIEB* gene codes for a protein product which would be acidic (isoelectric point about 4.5) consisting of 252 amino acid residues (M_r 28 100). Both cysteine and tryptophan are absent.

Codon usage (Wang & Doi, 1986) in both genes is essentially 'average' for *B. subtilis* although several 'uncommon' codons are used at an increased frequency in the *spoIIIEA* gene.

The presence of several potential promoter structures which could be utilized by RNA polymerase containing σ^{29} suggests that the expression of *spoIIIE* might be dependent on the processed *spoIIG* gene product (Trempey *et al.*, 1985). This could be tested experimentally, as could a requirement for the sigma-factor-like gene product of the *spoIIAC* gene (Errington *et al.*, 1985). Determination of the size of the *spoIIIE* message detected at t_3 suggests that a transcription-termination signal is close to the end of the region that was sequenced. An unusual feature of the *spoIIIE* operon is that the gap between the two genes is large – 50 bp including the termination codon of *spoIIIEA*. In other sporulation operons that have been sequenced the

reading frames are closer together or overlap (Fort & Errington, 1985). However a large gap, 36 bp, has been reported between the P23 and *dnaE* genes in the *B. subtilis* major sigma factor operon (Wang & Doi, 1986). The significance of such intercistronic regions which lack the potential to form secondary structures indicating potential RNA processing sites is not clear at present.

The requirement for *spoIIIE* gene products for the expression of three sporulation-related operons has been demonstrated. Mutations in *spoIIIE* prevent the expression of *spoVA* (Errington & Mandelstam, 1986), *gerA* (A. Moir, personal communication), and the 0.3 kb gene (Stephens *et al.*, 1984) as demonstrated by *lacZ* fusion studies. Expression of the *spoVA* and *gerA* operons has been shown to occur only in the spore compartment. Conversely, the expression of sporulation genes that are known to occur only in the mother cell compartment is not blocked by mutations in the *spoIIIE* operon. For example, the synthesis of dipicolinic acid (DPA) by DPA synthase, the product of the *spoVF* locus, is not dependent on *spoIIIE* expression (J. Errington & J. Mandelstam, unpublished results). This suggests that *spoIIIE* is part of a dependent sequence of operons expressed in the spore compartment. However, it is not yet known whether *spoIIIE* itself is expressed in the spore compartment or in the mother cell. The fact that protoplast fusion studies by Dancer & Mandelstam (1981) showed partial complementation of the *spoIIIE36* mutation by the wild-type suggests that it may be expressed in the mother cell with the implication that there is subsequently transport of the protein product(s) into the spore compartment. A more definite answer to the question should be obtainable by the use of *spoIIIE::lacZ* fusions.

No homology of amino acid sequences between the *spoIIIE* gene products and any known regulatory protein could be found (Protein Sequence Database, NIH). Also no sequences in either polypeptide showed any significant similarity to known DNA-binding proteins, i.e. they did not seem to have the so-called 'α-helix–turn–α-helix motif' (Pabo & Sauer, 1984). However, the failure to find such similarities does not rule out the possibility of a regulatory role for the proteins. Alternatively, the products of *spoIIIE* could have a physical effect on the assembly of spore components.

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