Studies of Transcriptional Regulation of the Bacillus subtilis Developmental Gene spoVE

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The structural gene of the spoVE locus of Bacillus subtilis was replaced with the promoterless lacZ gene of Escherichia coli. The spoVE::lacZ gene fusion was transferred to the B. subtilis chromosome and Q-galactosidase activity was measured under sporulation conditions. Expression of the hybrid gene could be detected as early as 40 min after the induction of sporulation. Transcription of the spoVE::lacZ gene was dependent on the products of two stage 0 loci, spo0H and spo0K. Mutations in the spoIIA and spoIIG loci did not impede expression of spoVE and, therefore, neither of the sigma factors coded for by these loci seems to be necessary for its transcription. Consequently, the spoVE locus does not seem to be part of the dependent sequence of operons involved in the developmental change, although its protein product is clearly needed for the completion of spore formation.

INTRODUCTION

The morphological changes of the later stages of sporulation in Bacillus subtilis may be separated in time from the underlying biochemical processes (Dion & Mandelstam, 1980; Jenkinson et al., 1980, 1981). Studies of the formation of the spore coat and the concomitant development of resistance properties have demonstrated that these are a result of self-assembly of proteins that are synthesized earlier. It is therefore not surprising to find that some stage V loci are expressed early in sporulation, i.e. well before the morphological changes characteristic of this stage are observed. Studies of transcriptional regulation of spoVG (Zuber & Losick, 1983) and spoVA (Savva & Mandelstam, 1985; Errington & Mandelstam, 1986) have revealed that these loci are transcribed at about t0 and t2.5, respectively. The results of these and many other experiments support the conclusion that almost all spo loci are expressed within 4 h of the onset of sporulation (Dion & Mandelstam, 1980; Jenkinson et al., 1980, 1981).

Cloning and sequencing of spoVE (Piggot et al., 1986; Bugaichuk & Piggot, 1986) provided data on the size and the structure of this locus. Its physiological function, however, cannot be understood without knowing when this gene is activated and to what extent its expression depends on other spo loci. To study this aspect of the role of spoVE in sporulation a promoterless copy of the Escherichia coli β-galactosidase gene, lacZ, was fused to the regulatory elements of spoVE. By measuring β-galactosidase activity in sporulating B. subtilis cells, I have now shown that the expression of the spoVE gene commences about 40 min after induction of sporulation and is prevented by mutations in two stage 0 loci.

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Abbreviation: MUG, 4-methylumbelliferyl β-D-galactoside.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Genotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>17</td>
<td>spoOH17 trpC2</td>
<td></td>
</tr>
<tr>
<td>43.2</td>
<td>spoA43 leu-8 rif-2 tal-1</td>
<td></td>
</tr>
<tr>
<td>55.3</td>
<td>spoIG55 trpC2</td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>spoI4A69 trpC2</td>
<td></td>
</tr>
<tr>
<td>MB75 (Spo+)</td>
<td>metC3 lys-1 tal-1</td>
<td>M. D. Yudkin, University of Oxford</td>
</tr>
<tr>
<td>87.2</td>
<td>spoOJ93 leu-8</td>
<td>Transformation of strain 168 with DNA from strain KS265 and selection for MLSR*</td>
</tr>
<tr>
<td>141</td>
<td>spo0K141 trpC2</td>
<td>This paper</td>
</tr>
<tr>
<td>168 (Spo+)</td>
<td>trpC2</td>
<td>Messing et al. (1981)</td>
</tr>
<tr>
<td>221.1</td>
<td>spo0F221 trpC2</td>
<td></td>
</tr>
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<td>484.2</td>
<td>spo0E11 trpC2</td>
<td></td>
</tr>
<tr>
<td>485.1</td>
<td>spo0G14 lys-1</td>
<td></td>
</tr>
<tr>
<td>486</td>
<td>spo0D8 trpC2</td>
<td></td>
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<td>488.4</td>
<td>spo0B136 trpC2</td>
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<td><strong>MY2000.63</strong></td>
<td>spoIAC63 lys-1 pyrD1 rif</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td><strong>KSV</strong></td>
<td>spoVG : : Tn917 trpC2 (MLS*)</td>
<td>This paper</td>
</tr>
<tr>
<td><strong>BS50</strong> (Spo+)</td>
<td>trpC2 spoVE : :lacZ</td>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<td>JM103</td>
<td>supE shcB15 strA thi endAΔ(lac-proAB) <a href="ZAM15">F' traD36 proAB lacI pneumonia, HisD45 araD15 endA1</a></td>
<td></td>
</tr>
<tr>
<td>JM107</td>
<td>endA1 gyr96 hsdR17 supE44 relA1 thi Δ(lac-proAB) <a href="ZAM15">F' traD36 proAB lacI pneumonia, HisD45 araD15 endA1</a></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>Relevant characteristics</td>
<td>Reference</td>
</tr>
<tr>
<td>pUC18</td>
<td>bla lacZ'</td>
<td>Norrander et al. (1983)</td>
</tr>
<tr>
<td>pSGMU32</td>
<td>bla lacZ cat</td>
<td>Errington (1986)</td>
</tr>
<tr>
<td>pUB4</td>
<td>bla; 1.35 kbp XhoI fragment of spoVE cloned in SalI site of pUC9</td>
<td>Bugaichuk &amp; Piggot (1986)</td>
</tr>
<tr>
<td>pUB5</td>
<td>bla spoVE : :lacZ'; deletion of 500 bp XhoI fragment of pUB4</td>
<td></td>
</tr>
<tr>
<td>pVL3</td>
<td>bla; 800 bp fragment of spoVE locus inserted in pUC18</td>
<td>This paper</td>
</tr>
<tr>
<td>pVL50</td>
<td>bla cat spoVE : :lacZ</td>
<td></td>
</tr>
<tr>
<td>pLM14</td>
<td>bla cat spoVE : :lacZ</td>
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</tbody>
</table>

* DNA from strain KS265 was a gift from K. Sandman (Harvard University, USA). MLSR*, resistance to macrolide, lincosamide and streptogramin B antibiotics.

**METHODS**

**Bacterial strains.** These are listed in Table 1.

**Growth and sporulation.** *B. subtilis* cells were grown in hydrolysed casein medium (CH) and induced to sporulate as described by Sterlini & Mandelstam (1969).

**Transformation.** The *B. subtilis* strains were transformed by the method of Anagnostopoulos & Spizizen (1961). Transformants were selected on Oxoid nutrient agar (NA) plates supplemented with chloramphenicol (Cm) at a concentration of 5 μg ml⁻¹. Strain KSV was selected on NA containing erythromycin, 1 μg ml⁻¹, and lincomycin, 25 μg ml⁻¹. For rapid detection of β-galactosidase activity on plates Schaeffer’s agar (Schaeffer et al., 1965) containing 30 μg 4-methylumbelliferyl β-D-galactoside (MUG) ml⁻¹ (Youngman et al., 1985a, b) was used. *E. coli* strains were transformed by the method of Hanahan (1983). Strains were maintained on LB agar; chloramphenicol (Cm) or ampicillin (Ap) were added, when necessary, at concentrations of 10 μg ml⁻¹ and 50 μg ml⁻¹, respectively. To detect expression of the lacZ gene, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal) was added to media at a concentration 40 μg ml⁻¹.
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Plasmids. Plasmids and their sources are described in Table 1. Plasmids were isolated from *E. coli* as described by Birnboim & Doly (1979). Small-scale rapid screening of recombinant plasmids was performed as described by Maniatis et al. (1982).

β-Galactosidase assays. *B. subtilis* cells harbouring the spoVE::lacZ fusion were grown and induced to sporulate as described by Sterlini & Mandelstam (1969). Samples were taken at intervals, centrifuged and quickly frozen. After thawing at room temperature cells were washed and resuspended in Z buffer (1 ml), toluenized and assayed for β-galactosidase activity as described by Miller (1972). One unit of specific β-galactosidase activity is defined as the amount of enzyme that releases 1 nmol o-nitrophenol min⁻¹ at 28 °C.

Construction of plasmid pVL3. Plasmid pUB5, which contains a translational spoVE::lacZ′ gene fusion (Bugaichuk & Piggot, 1986), was digested with *PstI* and treated with T4 DNA polymerase to blunt 3′ protruding cohesive ends. Plasmid DNA was purified and a second digestion with *AvaI* was carried out. The products of the reaction were separated by electrophoresis on 0.5%, low-melting-point agarose and the 0.8 kbp fragment of the spoVE gene was isolated and purified. This fragment was inserted into plasmid pUC18 prepared in the same way as described above, except that *SsrI* was used to linearize the plasmid. The new plasmid was propagated in *E. coli* strain JM103 and designated pVL3.

Treatment of DNA with Bal31. Fragments of the spoVE locus to be used for constructing transcriptional spoVE::lacZ fusions (Fig. 1) were prepared as follows. Plasmid pUB4 was cleaved at its unique *NotI* site and treated with *Bal31* (BRL) to reduce the N-terminal coding portion of the spoVE gene to a desirable size. The conditions of the reaction were adjusted to set the rate of DNA degradation at about 100 bp min⁻¹ at each end. The reaction was stopped by plunging the tube into liquid nitrogen. Treated DNA was purified with phenol and precipitated in ethanol. To increase the number of blunt ends the DNA was treated with the Klenow fragment of *E. coli* DNA polymerase I. The residual DNA encoding the C-terminal region of the spoVE gene was removed by *BamHI* digestion.

**RESULTS**

Construction of transcriptional spoVE::lacZ gene fusions

To study the regulation of the spoVE gene the method of ‘gene fusion’ was used to put the *E. coli* β-galactosidase gene (lacZ) under the control of the spoVE regulatory signals.

The transcriptional spoVE::lacZ fusions were constructed *in vitro* as outlined in Fig. 1. The plasmid pVL3 was linearized with *SmaI* and *PstI* endonucleases and purified by electrophoresis on 0.8%, low-melting-point agarose. This DNA was ligated with the 5.1 kbp *SmaI–PstI* fragment of pSGMU32, in which the lacZ gene had been altered by in-frame insertion of the translation initiation start derived from the spoIIA gene (Errington, 1986). Ligated DNA was transformed into *E. coli* strain JM107 [F⁻]. The pool of Ap⁸ transformants was screened for the Lac+ phenotype and several positive clones were selected for further analysis. All of them were found to contain the same type of plasmid, which was designated pVL50. The orientation of the insert in relation to the regulatory region of the spoVE gene was confirmed by restriction analysis. Construction of the plasmid pLM14 was similar to that of pVL50 except that a smaller fragment of spoVE was used (see Fig. 1).

Transfer of the spoVE::lacZ fusions to the *B. subtilis* chromosome

Plasmids pVL50 and pLM14 cannot replicate autonomously in *B. subtilis* since they lack a functional origin of replication. However, the presence of spoVE fragments within them makes it possible for these plasmids to become integrated into the chromosome by a Campbell-type mechanism. Bacteria with an integrated plasmid can be isolated by selecting for resistance to chloramphenicol. Transformation of *B. subtilis* strains 168 and MB75 (both Spo⁰) by these plasmids gave rise to chloramphenicol-resistant transformants with a Spo⁺ phenotype. The appearance of transformants with such a phenotype indicates that the chromosomal fragments of plasmids pVL50 and pLM14 contain a transcription-initiation site capable of directing the expression of the spoVE transcriptional unit (Piggot et al., 1984). Fifty Cm⁺ clones from each transformation were subcultured on NA + Cm, and then transferred to Schaeffer’s agar supplemented with MUG. After incubation for 24 h at 37 °C the vast majority of colonies derived from the transformations with pVL50 and LM14 were brightly fluorescent when viewed under UV light.
Fig. 1. Schematic representation of transcriptional spoVE::lacZ fusions. Part (a) depicts the construction of plasmids pVL50 and pLM14 (the scale is approximate). Regions coding for β-lactamase (bla), chloramphenicol resistance (cat) and β-galactosidase (lacZ) are shown as open boxes with the pointed end indicating the direction of transcription and translation. lacZ' corresponds to a region of DNA encoding only the amino-terminal portion of β-galactosidase. Only relevant restriction sites are shown. Treatment with the Klenow fragment of DNA polymerase I is indicated as 'Klenow'. ‘Bal31’ refers to the removal of double-stranded DNA with Bal31 exonuclease. Part (b) shows the junction area of transcriptional spoVE::lacZ fusions. The topmost line shows a restriction map of the spoVE locus. The open box indicates the location and length of the structural part of the spoVE gene; the arrow shows the direction of transcription. Fragments of the spoVE gene used for construction of transcriptional fusions are depicted as solid lines. Restriction sites: Av, AvaI; B, BamHI; Bg, BglII; H, HindIII; Nc, NcoI; Nr, NruI; P, PstI; S, SstI; Sm, Smal; X, XhoI. The lac–cat fragment was derived from plasmid pSGMU32 by digestion with enzymes Smal and PstI or Smal and BglII. The lacZ gene and the cat gene are shown as an open box and a hatched box, respectively. The spoIIA fragment containing the ribosome-binding site of ORF1 is shown as a filled box in front of lacZ. The zig-zag line denotes non-coding DNA of plasmid origin.
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3.35 kbp

SrSr
Sr

3.3 kbp

lacZ

SstI

Fig. 2. Schematic representation of the insertion of a transcriptional spoVE::lacZ fusion into the B. subtilis chromosome. The upper part of the figure shows the structure of plasmid pVL50, carrying a transcriptional spoVE::lacZ fusion. Below is depicted the restriction map of the area around the spoVE locus. Integration of pVL50 introduces an additional SsrI site. Predicted sizes for the new SsrI fragments are given in the bottom part of the figure. Restriction sites: Av, AvaI; Nr, NruI; S, SstI; Sm, SmaI; X, XhoI.

In order to confirm that a Campbell-type integration had taken place, chromosomal DNA from several CmR clones was analysed by Southern blotting. The DNA of CmR clones resulting from crosses of strain 168 with pVL50 was digested to completion with SsrI, separated on 0.7% agarose and blotted onto a nitrocellulose membrane. Blots were hybridized to the 4.9 kbp BamHI fragment of pSGMU23 (Errington, 1986) labelled with $^{32}$P. This probe hybridized strongly to two fragments, 3.3 and 8.5 kbp in size. These observations agree well, within experimental error, with insertion of plasmid pVL50 by a Campbell mechanism as outlined in Fig. 2.

During these experiments about 20% of the CmR clones generated by transformation of strain 168 with plasmid pVL50 were found to have an altered pattern of hybridization, with only an 8.5 kbp fragment appearing in SsrI-digested DNA. Similar observations were made during experiments to define the size of transcriptional unit of the spoVE locus (Piggot et al., 1986). The reasons for the appearance of such clones are not clear and they were excluded from subsequent experiments.

Expression of the spoVE::lacZ fusion under developmental control

Having established that strain BS50 (transcriptional fusion) contained the appropriate plasmid insertion, I set out to study the expression of the spoVE gene in sporulating B. subtilis cells. The results of these experiments are presented in Fig. 3. Strain BS50 was grown in CH medium and induced to sporulate by transfer to resuspension medium. Samples were collected at appropriate time intervals and $\beta$-galactosidase activity was assayed.

The activity of $\beta$-galactosidase in strain BS50 appeared to be considerably higher than the non-specific $\beta$-galactosidase background activity found in the parental strains MB75 and 168. Significant synthesis of $\beta$-galactosidase began about 40 min after the induction of sporulation. The activity continued to increase for another 5 h, reaching a peak between $t_5$ and $t_6$ and exceeding the background level by almost 10 times (Fig. 3).

The activity of $\beta$-galactosidase in a strain carrying an integrated copy of plasmid pLM14 was determined in analogous experiments and was found to be similar to that of strain BS50 (data not shown).
Fig. 3. spoVE-regulated expression of the lacZ gene in B. subtilis. β-Galactosidase activity was monitored in strain BS50, which carries a transcriptional spoVE::lacZ fusion (pVL50; ○). The parental strain 168 (contains no fusion; ▐) was assayed to determine non-specific background β-galactosidase activity. Strains were induced to sporulate by the method of Sterlini & Mandelstam (1969) and β-galactosidase activity was measured at hourly intervals as described by Miller (1972).

Fig. 4. Effect of mutations in the spo0A (▲), spo0B (■), spo0H (□) and spo0K (▲) loci on the expression of spoVE::lacZ. spo0 mutants bearing the transcriptional spoVE::lacZ fusion were grown and induced to sporulate as described by Sterlini & Mandelstam (1969).

Table 2. Effect of mutations on various spo loci on expression of the spoVE gene

The transcriptional spoVE::lacZ fusion was transferred into the appropriate genetic background by transformation and cells containing a chromosomal insertion were tested for β-galactosidase activity as described in Methods. Assays were performed at least twice with two independently isolated strains. +, Activity reaching 30% or more of that detected in the Spo+ background; −, always less than 10%.

<table>
<thead>
<tr>
<th>Mutated locus</th>
<th>β-Galactosidase activity</th>
<th>Mutated locus</th>
<th>β-Galactosidase activity</th>
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</thead>
<tbody>
<tr>
<td>spo0A</td>
<td>+</td>
<td>spo0H</td>
<td>−</td>
</tr>
<tr>
<td>spo0B</td>
<td>+</td>
<td>spo0J</td>
<td>+*</td>
</tr>
<tr>
<td>spo0D</td>
<td>+</td>
<td>spo0K</td>
<td>−</td>
</tr>
<tr>
<td>spo0E</td>
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<td>+</td>
</tr>
<tr>
<td>spo0G</td>
<td>+</td>
<td>spo0VG</td>
<td>+</td>
</tr>
</tbody>
</table>

* β-Galactosidase activity was 32% and 95% in strains 93.2 (spo0J93) and 87.2 (spo0J87) respectively.

Effect of mutations in various spo loci on transcription of the spoVE gene

The finding that expression of the spoVE gene can be detected as early as 40 min after the induction of sporulation prompted an examination of its possible dependence on the spo0 loci, which are known to be responsible for the initiation of development.

DNA extracted from strain BS50, harbouring the transcriptional spoVE::lacZ fusion, was introduced into several spo0 mutants by transformation, and CmR clones were screened for the Lac phenotype on Schaeffer’s agar supplemented with MUG. (During these experiments about 90% of the CmR clones were found to carry the lacZ gene.) To assay quantitatively the level of expression of the hybrid spoVE::lacZ gene in various mutants, β-galactosidase activity was determined using o-nitrophenyl β-D-galactoside as a substrate. The results showed that mutations in spo0K or spo0H loci abolished the synthesis of β-galactosidase, but mutations in
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spo0A, spo0B, spo0D, spo0E, spo0F or spo0G reduced the level of β-galactosidase activity by about 70% (Table 2, Fig. 4). Two mutations in the spo0J locus differed consistently in their effect on the expression of the spoVE::lac2 fusion. Mutation spo0J93 reduced the level of β-galactosidase activity by about 70%, but mutation spo0J87 had little effect on the synthesis of β-galactosidase. Mutations in spoIIA, spoIIG or spoVG were without significant effect.

DISCUSSION

The view that the morphological changes which occur late in spore formation in *B. subtilis* could be the result of interactions between components synthesized much earlier is receiving experimental support as the number of cloned and characterized 'late' loci increases. Several stage V loci (spoVG, spoVK, spoVM and spoVN) begin to be transcribed within 2 h of the onset of sporulation (Losick et al., 1986), and transcription of the spoVA operon begins at about t2.5 (Savva & Mandelstam, 1985, 1986; Errington & Mandelstam, 1986). My results have now shown that the spoVE locus is also transcribed several hours before its product is believed to be required.

By constructing the transcriptional spoVE::lac2 fusions (plasmids pVL50 and pLM14) I was able to determine more precisely the time at which expression of this locus begins. In these gene fusions, synthesis of the hybrid protein with β-galactosidase activity is initiated from a translational start point derived from another developmental *B. subtilis* gene, spoUAA (Errington, 1986). As can be seen from the data presented in Fig. 3, transcription of the spoVE gene started about 40 min after sporulation was initiated and continued until at least t5. These results, coupled with the observation that β-galactosidase activity during the exponential phase of growth does not exceed the background level, indicate that the time of spoVE induction is determined by the regulatory system of sporulation. They also suggest that transcription of the spoVE gene occurs well before asymmetric septation (stage II) is completed; unless expression of the gene involves post-transcriptional control it follows that the protein product may be present not only in the mother cell (de Lencastre & Piggot, 1979) but also in the forespore. If that is the case, one could explain why previous attempts to obtain complementation of the spoVE locus by protoplast fusion were unsuccessful (Dancer & Mandelstam, 1981). It could be argued that at the time when protoplast fusion was carried out (t3), a faulty spoVE product had already interacted irreversibly with some other component(s) and addition of the undamaged spoVE product occurred too late to restore the sporulation sequence.

By introducing the transcriptional spoVE::lacZ fusion into nine stage 0 mutants I was able to examine the effect of the spo0 genes on the induction of spoVE. Mutations in the spo0H and spo0K loci almost entirely blocked production of β-galactosidase activity; the remaining stage 0 mutations had less effect: the level of β-galactosidase was reduced to about 30% by mutations in spo0A, spo0B, spo0D, spo0E, spo0F or spo0G (Fig. 4, Table 2). While mutation spo0J93 reduced the level of β-galactosidase activity to about 30%, mutation spo0J87 had very little effect (Table 2). This is in keeping with the results of Clarke & Mandelstam (1987), who also found that this latter mutation blocked development but allowed expression of other sporulation operons.

It has previously been pointed out that the area lying upstream from the translation initiation site of the spoVE gene shows homology with the spoVG promoters (Banner et al., 1983; Bugaichuk & Piggot, 1986). Analysis of the spo0-dependent regulation of the spoVE transcription activity points to further similarities between these two genes. All the spo0 loci except spo0A and spo0B affect transcription of spoVE and spoVG in a similar fashion. In the absence of a clear understanding of the functions of the spo0A and spo0B loci it is difficult to assess the significance of the dependence of spoVE transcription on these genes. Dependence of the two genes on the spo0H locus may be significant. The recently discovered homology with known sigma factors suggests that the product of the spo0H gene is a new form of sigma specis (I. Smith, personal communication). This conclusion agrees quite well with the proposed role of spo0H in transcriptional regulation of the spoVG gene (Zuber & Losick, 1985). It seems plausible that the spo0H product may interact with the spoVE promoter(s) in a similar way.
To investigate the possible role of the $\sigma^{29}$ factor (Trempy et al., 1985) and what is currently known as the sigma-IIAC factor (Errington et al., 1985; Yudkin, 1987) in expression of spoVE, the hybrid spoVE::lacZ gene was transferred to strains 55.3 (spoIIG), 69 (spoIIA69) and MY2000.63 (spoIAC63). The pattern of $\beta$-galactosidase expression in these strains was not found to be significantly different from that in the wild-type. This observation agrees with available data on transcriptional activation of the spoIIG and spoIHA loci (reviewed by Doi & Wang, 1986) and indicates that the initiation of transcription of the spoVE gene does not require participation of these sigma factors.

Four of the stage V loci, spoVA, spoVC, spoVD and spoVF, are in a line of expression dependent on spoIHA, spoIIG, etc. (Holland et al., 1987). By contrast, spoVE and spoVGC do not seem to part of this dependent sequence of gene expression. Clearly, more information about the function of these loci is needed before we can interpret dependence relationships in terms of molecular interactions, which are the essence of the sporulation process.

I am grateful to Professor J. Mandelstam for his support and invaluable encouragement throughout this research project and to Dr M. D. Yudkin for critical reading of the manuscript and advice. I thank Dr J. Errington for donation of plasmids and Mr D. Ramsay for the art work. This work was supported by the Science and Engineering Research Council.

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