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 β -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 t_0 and $t_{2.5}$, 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*

Bacteria	Genotype	Source/reference
<i>B. subtilis</i>		
17	<i>spo0H17 trpC2</i>	Laboratory stock
43.2	<i>spo0A43 leu-8 rif-2 tal-1</i>	
55.3	<i>spoIIG55 trpC2</i>	
69	<i>spoIIAA69 trpC2</i>	
MB75	(Spo ⁺) <i>metC3 lys-1 tal-1</i>	
87.2	<i>spo0J87 leu-8</i>	
93.2	<i>spo0J93 trpC2</i>	
141	<i>spo0K141 trpC2</i>	
168	(Spo ⁺) <i>trpC2</i>	
221.1	<i>spo0F221 trpC2</i>	
484.2	<i>spo0E11 trpC2</i>	
485.1	<i>spo0G14 lys-1</i>	
486	<i>spo0D8 trpC2</i>	
488.4	<i>spo0B136 trpC2</i>	
MY2000.63	<i>spoIIAC63 lys-1 pyrD1 rif</i>	M. D. Yudkin, University of Oxford
KSV	<i>spoVG::Tn917 trpC2 (MLS^R)</i>	
BS50	(Spo ⁺) <i>trpC2 spoVE::lacZ</i>	Transformation of strain 168 with DNA from strain KS265 and selection for MLS ^R *
<i>E. coli</i>		
JM103	<i>supE sbcB15 strA thi endAΔ(lac-proAB) [F' traD36 proAB lacI^s ZΔM15]</i>	Messing <i>et al.</i> (1981)
JM107	<i>endA1 gyrA96 hsdR17 supE44 relA1 thi Δ(lac-proAB) [F' traD36 proAB lacI^s ZΔM15]</i>	Yanisch-Perron <i>et al.</i> (1985)
Plasmids	Relevant characteristics	Reference
pUC18	<i>bla lacZ'</i>	Norrande <i>et al.</i> (1983) Errington (1986)
pSGMU32	<i>bla lacZ cat</i>	
pUB4	<i>bla</i> ; 1.35 kbp <i>XhoI</i> fragment of <i>spoVE</i> cloned in <i>Sall</i> site of pUC9	Bugachuk & Piggot (1986)
pUB5	<i>bla spoVE::lacZ'</i> ; deletion of 500 bp <i>AvaI</i> fragment of pUB4	
pVL3	<i>bla</i> ; 800 bp fragment of <i>spoVE</i> locus inserted in pUC18	This paper
pVL50	<i>bla cat spoVE::lacZ</i>	
pLM14	<i>bla cat spoVE::lacZ</i>	

* DNA from strain KS265 was a gift from K. Sandman (Harvard University, USA). MLS^R, 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.

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 (Bugachuk & Piggot, 1986), was digested with *Pst*I and treated with T4 DNA polymerase to blunt 3' protruding cohesive ends. Plasmid DNA was purified and a second digestion with *Ava*I 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 *Ssr*I was used to linearize the plasmid. The new plasmid was propagated in *E. coli* strain JMI03 and designated pVL3.

Treatment of DNA with *Bal*31. 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 *Nco*I site and treated with *Bal*31 (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 *Bam*HI 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 *Sma*I and *Pst*I endonucleases and purified by electrophoresis on 0.8% low-melting-point agarose. This DNA was ligated with the 5.1 kbp *Sma*I-*Pst*I fragment of pSGMU32, in which the *lacZ* gene had been altered by in-frame insertion of the translation initiation start derived from the *spoIIAA* gene (Errington, 1986). Ligated DNA was transformed into *E. coli* strain JM107 [F⁻]. The pool of Ap^R 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 Spo⁺) 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^R 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, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; Nc, *Nco*I; Nr, *Nru*I; P, *Pst*I; S, *Sst*I; Sm, *Sma*I; X, *Xho*I. The *lac*-*cat* fragment was derived from plasmid pSGMU32 by digestion with enzymes *Sma*I and *Pst*I or *Sma*I and *Bgl*II. 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.

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 *SstI* site. Predicted sizes for the new *SstI* 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 Cm^R clones was analysed by Southern blotting. The DNA of Cm^R clones resulting from crosses of strain 168 with pVL50 was digested to completion with *SstI*, separated on 0.7% agarose and blotted onto a nitrocellulose membrane. Blots were hybridized to the 4.9 kbp *Bam*HI 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 Cm^R 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 *SstI*-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 β -galactosidase activity was assayed.

The activity of β -galactosidase in strain BS50 appeared to be considerably higher than the non-specific β -galactosidase background activity found in the parental strains MB75 and 168. Significant synthesis of β -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 β -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

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

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%.

Mutated locus	β -Galactosidase activity	Mutated locus	β -Galactosidase activity
<i>spo0A</i>	+	<i>spo0H</i>	-
<i>spo0B</i>	+	<i>spo0J</i>	+*
<i>spo0D</i>	+	<i>spo0K</i>	-
<i>spo0E</i>	+	<i>spoIIA</i>	+
<i>spo0F</i>	+	<i>spoIIG</i>	+
<i>spo0G</i>	+	<i>spoVG</i>	+

* 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 *Cm*^R clones were screened for the Lac phenotype on Schaeffer's agar supplemented with MUG. (During these experiments about 90% of the *Cm*^R clones were found to carry the *lacZ* gene.) To assay quantitatively the level of expression of the hybrid *spoVE::lac* 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

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::lacZ* 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 $t_{2.5}$ (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::lacZ* 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, *spoIIAA* (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 t_5 . 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 (t_3), 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 species (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 σ^{29} factor (Trempey *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 (*spoIIAA69*) and MY2000.63 (*spoIIAC63*). The pattern of β -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 *spoIIA* 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 *spoIIA*, *spoIIG*, etc. (Holland *et al.*, 1987). By contrast, *spoVE* and *spoVG* do not seem to be 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.

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