Use of *lacZ* Gene Fusions to Determine the Dependence Pattern of the Sporulation Gene *spoIID* in *spo* Mutants of *Bacillus subtilis*

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The *spoIID* gene, which is involved in *Bacillus subtilis* sporulation, was fused to the β -galactosidase gene, *lacZ*, of *Escherichia coli* so that the expression of β -galactosidase would be under the control of the *spoIID* locus. When the fused product was inserted into the *B. subtilis* chromosome, production of β -galactosidase indicated that the *spoIID* gene was expressed 1.5 h after the start of sporulation. When the *spoIID::lacZ* fusion was inserted into the chromosome of sporulation mutants, all strains carrying *spo0* lesions and those with mutations in *spoIIA*, *spoIIE* and *spoIIG* loci failed to make β -galactosidase. The proposed provisional order of expression of operons governing stage II is *spoIIA* $\rightarrow$ [*spoIIG*, *spoIIE*] $\rightarrow$ [*spoIID*, *spoIIB*, *spoIIF*].

INTRODUCTION

The initiation of sporulation in *Bacillus subtilis* is morphologically characterized by the deposition of an asymmetric septum. This is conventionally called stage II of the process, which in *B. subtilis*, is controlled by six known genetic loci: *spoIIA*, *spoIIB*, *spoIID*, *spoIIE*, *spoIIF* and *spoIIG*. A seventh postulated locus, *spoIIC*, has been recently shown to be the same as *spoIID* (Lopez-Diaz *et al.*, 1986). Mutations in any of the stage II genetic loci block further steps in sporulation and may also give rise to a variety of bizarre cell forms.

Methods have been described (Errington, 1986) for fusing the β -galactosidase gene, *lacZ*, of *Escherichia coli* into sporulation operons so that enzyme production is determined either by the transcriptional or by the translational control system of the sporulation operon. The fused product can be inserted into a variety of genetic backgrounds by transformation, and production of the enzyme can then be measured either in *Spo*⁺ strains or in strains carrying a mutation in any one of a number of different sporulation operons.

The experiments described here were intended to obtain at least partial answers to the following questions. (a) At what time is the *spoIID* operon turned on? (b) Which sporulation operons determine whether or not *spoIID* will be expressed? (c) Is there an appreciable time lag between the time the message appears and the time at which it is translated? If there were such a gap, translational control would enter as an additional regulatory factor in sporulation. It was for this reason that both transcriptional and translational *lacZ* fusion plasmids were constructed.

METHODS

Bacterial strains, phages and plasmids. These are listed in Table 1.

Buffers, chemicals and solutions. The ligation buffer was 66 mM-Tris/HCl (pH 7.2), 5 mM-MgCl₂, 5 mM-DTT, 1 mM-ATP. Tris buffer was 50 mM-Tris/HCl (pH 7.5). Z buffer (pH 8) contained Na₂HPO₄·7H₂O (60 mM), NaH₂PO₄·H₂O (40 mM), KCl (10 mM), MgSO₄·7H₂O (1 mM), 2-mercaptoethanol (50 mM). RE buffer contained Tris/HCl (50 mM, pH 7.6), KCl (50 mM), MgCl₂ (10 mM), DTT (10 mM). MUG is 4-methylumbelliferyl- β -D-

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Table 1. *Plasmids, bacteriophages and bacterial strains*

Relevant properties		Source
Plasmid		
pSG28	Transcriptional <i>lac</i> fusion vector	Errington (1986)
pSG23	Translational <i>lac</i> fusion vector	
pSGLD1	pUC13 + 4 kbp <i>EcoRI</i> <i>spoIID</i> locus	Lopez-Diaz <i>et al.</i> (1986)
pSGLD4	1.1 kbp <i>HindIII</i> fragment in pSGMU2	
pSGLD8	0.6 kbp <i>AvaI</i> - <i>HindIII</i> in pSGMU2	
Phage		
φ105LD2	<i>spoIID</i> ⁺ , 2 kbp <i>MboI</i> insert in φ105J9	Lopez-Diaz <i>et al.</i> (1986)
<i>B. subtilis</i>	Genotype	Source
<i>Spo</i> ⁺ strains		
168	<i>trpC2</i>	Laboratory stock
MB75	<i>lys-1 metC3 tal-1</i>	
SG46	<i>lys-1 metC3 tal-1 spoIID::lacZ-cat</i>	This paper
SG47	<i>lys-1 metC3 tal-1 spoIID::lacZ-cat</i>	
<i>Spo</i> ⁻ strains*		
43.2	<i>spo0A43 leu-8</i>	NG6.21; Piggot (1973)
488.1	<i>spo0B136 lys-1</i>	<i>spo0B136</i> ; Hoch & Mathews (1973)
485.1	<i>spo0G485 lys-1</i>	14UL; Ionesco <i>et al.</i> (1970)
46	<i>spo0H46 trpC2</i>	NG7.17; Piggot (1973)
87.2	<i>spo0J87 leu-8</i>	Hranueli <i>et al.</i> (1974)
69.1	<i>spoIIA69 trpC2</i>	NG18.6; Piggot (1973)
131.3	<i>spoIIB131 metC3</i>	Z3; Coote (1972a)
96	<i>spoIIF96 trpC2</i>	Hranueli <i>et al.</i> (1974)
55.2	<i>spoIIG55 trpC2</i>	NG12.12; Piggot (1973)
35.2	<i>spoIIIA35 metC3 tal-1 rif-2</i>	NG1.13; Piggot (1973)
26.2	<i>spoIIIA26 leu-8 tal-1</i>	E34; Waite <i>et al.</i> (1970)
590	<i>spoIIIF590 lys-1</i>	Lamont & Mandelstam (1984)
133.1	<i>spoIVC133 trpC2</i>	Z7; Coote (1972b)
498.2	<i>spoIVE498 trpC2</i>	IIT; Ionesco <i>et al.</i> (1970)
5	<i>spoIVG5 trpC2</i>	A8; Waite <i>et al.</i> (1970)
25.5	<i>spoIVG25 trpC2</i>	E33; Waite <i>et al.</i> (1970)
89.9	<i>spoVA89 lys-1</i>	Hranueli <i>et al.</i> (1974)
156.2	<i>spoVD156 trpC2</i>	W10; Coote (1972a)
153	<i>spoVE153 trpC2</i>	W5; Coote (1972a)

* Other sporulation mutants used were those listed by Errington & Mandelstam (1986a).

galactoside (Sigma). MU is 4-methylumbelliferone (Sigma). Chase solution was a mixture containing the four deoxyribonucleoside triphosphates (each at 0.5 mM). 'Klenow fragment' (Amersham) is the large fragment of *E. coli* DNA polymerase I.

Restriction endonuclease digestions. Restriction enzymes were obtained from Amersham or BRL and used as recommended by the suppliers.

Preparation of DNA. Plasmid DNA was extracted from *E. coli* by the alkaline lysis method (Maniatis *et al.*, 1982). If, after digestion with restriction endonuclease, the sticky ends were to be filled in, the DNA was phenol-extracted and precipitated in ethanol as described by Errington (1984). It was then resuspended to a volume of 30 µl in RE buffer, 4 µl chase solution, 0.5 units of 'Klenow fragment' and H₂O. After incubation for 10 min at room temperature, the Klenow polymerase was destroyed by heating the mixture at 70 °C for 10 min. When a DNA fragment was to be recovered from agarose gels, low melting point agarose (BRL) was used and the DNA was extracted by the method of Savva & Mandelstam (1984).

Chromosomal DNA for transformations was prepared according to Ward & Zahler (1973).

Growth of bacteria and induction of sporulation. Cells were grown at 37 °C in casein hydrolysate medium and induced to sporulate in minimal sporulation medium as described by Sterlini & Mandelstam (1969). The time at which cells were resuspended in sporulation medium is referred to as *t*₀ and hourly intervals thereafter as *t*₁, *t*₂, etc. The density of cultures at *t*₀ was approximately 0.24 mg dry weight per ml.

Transformation and selection of recombinants. *B. subtilis* recipient cells were made competent and transformed as described by Jenkinson (1983). Selection for chloramphenicol resistance was made on nutrient agar (Oxoid) containing chloramphenicol (5 µg ml⁻¹) and MUG (50 µg ml⁻¹), which allowed recognition of Lac⁺ colonies.

Transductions and infection with phage ϕ 105. Transduction to Spo⁺ and selection of transductants were as described previously (Lopez-Diaz *et al.*, 1986). Alternatively, a lawn of recipient cells was spread on a nutrient agar plate and drops (5 μ l) of phage lysate were spotted onto the lawn. Phage infection produced clearly visible lysis and surviving cells were streaked from the centre of plaques and tested for immunity to phage ϕ 105.

Measurement of β -galactosidase activity. This was done as described by Errington & Mandelstam (1986*a*) except that culture samples were 1 ml instead of 0.5 ml and Z buffer was adjusted to pH 8 instead of pH 7.

*Construction of *B. subtilis* strains SG46 and SG47.* For the construction of strain SG46, plasmid pSG28 was digested with *Sma*I and *Bgl*II and the 4.8 kbp *lacZ-cat* fragment was recovered from a low melting point gel, plasmid pSGLD4 was opened with *Bam*HI, and plasmid pSGLD1 was digested with *Ava*I and the ends filled in. Equimolar amounts of the three pieces of DNA, to give a total of approximately 3 μ g, were ligated together and used to transform strain MB75 to chloramphenicol resistance. Strain SG47 was made in a similar way using plasmid pSG23 cut with *Sma*I and *Bgl*II, pSGLD4 opened with *Bam*HI, and pSGLD8 cut with *Hind*III and the ends filled in. In each case a Cm^R Spo⁻ Lac⁺ recombinant colony of MB75 was isolated and purified. The Spo⁺ *lac* fusion strains were made by transducing the Spo⁻ strains to Spo⁺ with phage ϕ 105LD2, a recombinant phage containing the *spoIID* locus (Lopez-Diaz *et al.*, 1986).

RESULTS

Construction of the transcriptional lac fusion

The transcriptional *lac* fusion vector plasmid pSG28 (Errington, 1986) contains a 4.8 kbp *lacZ-cat* fragment flanked on both sides by useful restriction sites which allow the *lacZ* gene to be removed without a promoter but with appropriate *B. subtilis* translation signals. To construct a *spoIID::lacZ* fusion, plasmid pSG28 was digested with *Sma*I and *Bgl*II, and the 4.8 kbp *lacZ-cat* fragment was recovered from a low melting point agarose gel. This DNA was then ligated to fragments of the *spoIID* transcriptional unit so that the *lacZ-cat* fragment would be flanked by two regions of the *spoIID* locus, one of which is known to contain the *spoIID* promoter. The details of the construction are given below, and are shown in Fig. 1.

Lopez-Diaz *et al.* (1986) described the construction of several plasmids containing fragments of the *spoIID* region. One of these, plasmid pSGLD1, a derivative of plasmid pUC13, contains the entire *spoIID* transcriptional region in an *Eco*RI fragment. Plasmid pSGLD1 was cut with *Ava*I and the 'sticky' ends were filled in using 'Klenow' polymerase. Previous results using integrational plasmids (Lopez-Diaz *et al.*, 1986) showed that *Ava*I would separate the *spoIID* promoter region from the translated region. *Ava*I would also cut at the *Sma*I site in the polylinker of pUC13 – thus two blunt ended fragments would be produced (Fig. 1). Plasmid pSGLD4, an integrational plasmid carrying Cm^R and which contains the end of the *spoIID* transcriptional unit (Lopez-Diaz *et al.*, 1986), was opened with *Bam*HI (at a site in the polylinker of the original integrational plasmid vector) to provide the second *spoIID* flanking region.

The two opened plasmids and the *lacZ-cat* fragment were ligated together (a total of about 3 μ g DNA) and used to transform *B. subtilis* MB75, which was then plated on agar containing chloramphenicol. Typically, only about 20 Cm^R colonies were obtained. The desired Cm^R colonies result from ligation of the fragments shown in Fig. 1, followed by recombination between both flanking fragments and the *B. subtilis* chromosome to insert the *lacZ-cat* fragment into the *spoIID* locus. Some of the Cm^R colonies could have resulted from self-ligation of the Cm^R plasmid pSGLD4 followed by integration into the chromosome. However, as shown in previous work (Lopez-Diaz *et al.*, 1986), such integrations yield only Spo⁺ Cm^R colonies, since pSGLD4 contains the end of the *spoIID* transcription unit. About 20% of transformants were Spo⁺.

A Cm^R Spo⁻ Lac⁺ colony was selected, purified and named SG46. This strain was found to be stable, i.e. no Spo⁺ or Cm^S segregants were detected. Donor DNA from strain SG46 was able to transform recipient strain MB75 to Cm^R and all Cm^R transformants were Spo⁻ and Lac⁺. The Spo⁻ phenotype of the donor and recipient strains was complemented by the recombinant phage ϕ 105LD2, which contains the *spoIID* locus (Lopez-Diaz *et al.*, 1986).

Construction of the translational lac fusion

The translational *lac* fusion plasmid pSG23 (Errington, 1986) contains a 4.8 kbp *lacZ-cat* region with a series of restriction enzyme sites inserted three codons after the start of the *lacZ*

Fig. 1. Schematic representation of the method used to construct the transcriptional *lac* fusion strain SG46 by insertion of the *lacZ-cat* fragment from plasmid pSG28 (Errington, 1986) into the *spoIID* gene. Plasmids pSGLD1 and pSGLD4, which contain fragments of the *spoIID* region (Lopez-Diaz *et al.*, 1986), and pSG28 containing the *lacZ-cat* fragment were digested with restriction enzymes as shown. The *AvaI* sticky ends were filled in and the fragments were ligated together. The ligated DNA was used to transform strain MB75 and chloramphenicol resistant colonies were selected. Those which were also *Spo*⁻ and *Lac*⁺ were picked., *In vitro* ligation; ----, the extent of the homologous regions where crossover can occur; —, *B. subtilis* chromosomal DNA; ~~~, plasmid DNA; ▨, *lacZ* gene; ■, *cat* gene; □, *spoIID* gene.

gene. Thus the *lacZ-cat* region could be recovered, without its own transcription and translation signals, and ligated in frame to *spoIID* fragments so that β -galactosidase would be synthesized under the transcriptional and translational control of *spoIID*.

The construction of the *spoIID* translational *lac* fusion strain is outlined in Fig. 2. The *lac* fusion vector plasmid pSG23 was digested with *SmaI* and *BglII* to release the *lacZ-cat* fragment. The sequence of the *spoIID* gene (Lopez-Diaz *et al.*, 1986) showed that the *HindIII* site in the *AvaI-HindIII* fragment of plasmid pSGLD8 should, after filling in, give an in-frame fusion when ligated to the *lacZ* gene. Therefore, plasmid pSGLD8 was opened with *HindIII* and the ends were filled in. Plasmid pSGLD4 was opened with *BamHI* to provide the second *spoIID* fragment. The three were ligated together; the DNA mixture was used to transform MB75, and *Cm*^R transformants were selected. *Cm*^R transformants were expected to arise as a result of three different mechanisms: (1) by insertion of the *spoIID::lacZ* fusion into the chromosome (as shown in Fig. 2) by recombination with the homologous regions flanking the *lacZ-cat* fragment; (2) by integration of circular structures containing *spoIID::lac* fusions into the chromosome by a single crossover Campbell mechanism; (3) by integration of recircularized plasmids pSGLD8 or pSGLD4. Although *Cm*^R transformants of the *Spo*⁻ phenotype could result from any of these three mechanisms, only the first and the second would give a *Lac*⁺ phenotype. However, only transformants resulting from the first mechanism would be stable, since transformants arising from a single crossover would contain a duplication in the *spoIID* locus. This duplication would be expected to result in the segregation of *Cm*^S *Spo*⁺ *Lac*⁻ colonies under non-selective conditions when recombination between the duplicated regions eliminated the plasmid (Ferrari *et al.*, 1983).

A stable *Cm*^R *Spo*⁻ *Lac*⁺ transformant was isolated and named SG47. The *Spo*⁻ phenotype was complemented by phage ϕ 105LD2, indicating that the property is a result of the inactivation of the *spoIID* gene. DNA from SG47 was able to transform MB75 to *Cm*^R; all resistant colonies were *Spo*⁻ and *Lac*⁺.

Fig. 2. Schematic representation of the method used to construct the translational *lac* fusion strain SG47 by insertion of the *lacZ-cat* fragment from the translational fusion vector plasmid pSG23 (Errington, 1986) into the *spoIID* gene. Plasmids pSGLD8 and pSGLD4 which contain fragments of the *spoIID* region (Lopez-Diaz *et al.*, 1986), and the fusion plasmid pSG23 were digested with restriction enzymes as shown. The *Hind*III sticky ends were filled in and the fragments were ligated together and used to transform strain MB75. Chloramphenicol resistant colonies were selected and those which were *Spo*⁻ and *Lac*⁺ were picked. The symbols are defined in Fig. 1.

Measurement of β -galactosidase activity under sporulation conditions

The *lac* fusion strains SG46 and SG47 were transduced with phage ϕ 105LD2 in order to complement the disrupted *spoIID* gene and make the strains *Spo*⁺. The activity of β -galactosidase was then measured in these *Spo*⁺ strains at t_0 and at 30 min intervals after the induction of sporulation. The results are shown in Fig. 3. β -Galactosidase starts to be synthesized at about $t_{1.5}$ and reaches its maximum at $t_2-t_{2.5}$. The same pattern was present in both transcriptional and translational fusion strains.

Effect of sporulation mutations on the expression of *spoIID*

The *spoIID* translational *lac* fusion was transferred to the chromosome of a range of sporulation mutants in the following way. DNA from strain SG47 was used to transform competent cells of each mutant and *Cm*^R transformants were selected. These transformants would be double mutants in that they would now carry a disrupted *spoIID* gene as well as the original lesion. The *spoIID* mutation in each was then complemented by infecting the cells with recombinant phage ϕ 105LD2, which carries the *spoIID* locus. Each infected strain was tested for immunity to ϕ 105 (see Methods) and microscopically for the restoration of the original *Spo* phenotype in the case of those mutants blocked at later stages, i.e. stage III onwards. β -Galactosidase production was then measured at t_0 and at hourly intervals to t_4 or t_5 . In each experiment *B. subtilis* strains 168 and SG47 were used as negative and positive controls respectively. The results are shown in Table 2. No β -galactosidase activity was found in any stage 0 mutants or in any stage II mutants except *spoIIB* and *spoIIF*. β -Galactosidase was produced in all stage III, IV, and V mutants tested.

DISCUSSION

From work done in various laboratories in the past few years it is now apparent that most if not all of the sporulation operons controlling stage 0-I of sporulation are expressed during

Fig. 3. β -Galactosidase activity in the *spoIID* transcriptional fusion strain SG46 and the translational fusion strain SG47. Both strains were transduced with $\phi 105LD2$ (which contains the *spoIID* gene) to complement the disrupted gene in the fusion strains and make them *Spo*⁺. Samples (1 ml) were taken hourly, incubated with MUG and the fluorescent product measured against standards (see Methods). ●, SG46, the transcriptional *lac* fusion strain; △, SG47, the translational *lac* fusion strain; ○, *B. subtilis* 168 without a *lacZ* gene, showing a spurious low-level background activity.

Table 2. *Expression of the spoIID operon in different mutants*

The *lacZ* gene was transferred to strains harbouring defects in *spo* loci by transformation with DNA from strain SG47. The *spoIID* mutation in each was then complemented by infecting with the recombinant phage $\phi 105LD2$. The resulting strains were grown up, transferred to sporulation medium and the cultures sampled at hourly intervals for the measurement of β -galactosidase. Peak activity was generally found at t_2 , unless indicated otherwise. +, Activity roughly equal to that of the wild-type; -, activity about the same as the background activity of *B. subtilis* containing no *lacZ* gene (see Fig. 3); +*, about 50% of the activity of the wild-type; +**, about 50% activity but delayed by about 1 h.

Mutated locus	β -Galactosidase activity						<i>ger</i>
	0-I	II	III	IV	V	VI	
A	-	-	+	+	+	+	
B	-	+	+	+	+		
C			+	+	+	+	
D	-		+	+	+		
E	-	-	+	+	+		+
F	-	+	+	+	+		
G	-	-		+			
H	-				+		
J	-				+		
K	-						

vegetative growth. It has also long been known that mutations in some of the stage 0-I genes affect not only the ability to sporulate but also competence in genetic transformation, the response to infection by various types of bacteriophage, etc. In the light of this information it seems reasonable to exclude the stage 0-I operons from the sequence of events that is set in train when sporulation is initiated by subjecting cells of *B. subtilis* to conditions of nutritional 'step-down'.

On the basis of this view, sporulation as a developmental process can be considered to begin with stage II, i.e. with the laying down of a membrane septum asymmetrically to give two unequal compartments, the smaller of which is then engulfed by the larger and develops into a spore. The events from the formation of the septum to the engulfment are known to be specifically controlled by six operons (see Introduction) although it is possible that one or more operons still remain to be discovered.

Two questions posed in the Introduction are answered by the results illustrated in Fig. 3. It is apparent that the curves for transcription and translation both rise sharply at about t_2 and, within the accuracy of the method, there is no delay between them. Clearly this does not rule out translational control, but it does mean that it is a factor that need not be taken into consideration. Incidentally, results obtained for the *spoVA* operon (Savva & Mandelstam, 1985; Errington & Mandelstam, 1986*b*) indicate that there is no translational delay in its expression; by the time the message becomes measurable by Northern blot hybridization, its translation is already detectable by the more sensitive *lacZ* fusion technique. By contrast, kinetic measurements on the rate of disappearance of the enzyme suggest the possibility that there may, indeed, be a translational control factor (Errington & Mandelstam, 1986*a*).

It is apparent (see Table 2) that *spoIID* is not expressed in any strains carrying mutations in stage 0 operons. This merely confirms the point, made in the Introduction, that expression of the *spo0* genes during vegetative growth is a prerequisite for initiating sporulation.

spoIID failed to be expressed in strains that were damaged in *spoIIA*, *spoIIE* and *spoIIG*. The expression of these three loci can, therefore, be reasonably assumed to precede that of *spoIID*. It is known that the expression of *spoIIA* is not dependent on the intactness of any of the other loci governing stage II (Errington & Mandelstam, 1986*a*). This finding could by itself be interpreted in either of two ways: firstly that *spoIIA* is the earliest of the known stage II operons to be expressed, or alternatively that it is expressed in parallel with one or more stage II operons. With *spoIIE* and *spoIIG* we have, however, the additional information that *spoIIG* is not expressed in *spoIIA* mutants (Trempy *et al.*, 1985*a*). We also know that *spoIIG* codes for a pre-sigma factor (M_r 31 000), which may then be processed by *spoIIE* to give the sigma factor σ^{29} (Trempy *et al.*, 1985*b*). These facts would place *spoIIA* earlier in a linear sequence than either of the other two operons. We have no information at present that allows us to determine whether *spoIIE* and *spoIIG* are expressed together or in sequence. In the scheme given below they are therefore bracketed together.

The results of the present paper make it apparent that expression of *spoIID* follows that of the other three loci. Two others remain to be considered, *spoIIB* and *spoIIF*. According to the data of Table 2, these are clearly later in stage II than any of the others we have considered, and the provisional order of expression is therefore as follows: *spoIIA* $\rightarrow$ [*spoIIG*, *spoIIE*] $\rightarrow$ [*spoIID*, *spoIIB*, *spoIIF*].

Clearly the construction of further *lacZ* fusions can be expected to give an unequivocal order of expression of the loci controlling stage II of sporulation.

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