Use of a *lacZ* Gene Fusion to Determine the Dependence Pattern of Sporulation Operon *spoIIIC* in *spo* Mutants of *Bacillus subtilis*: a Branched Pathway of Expression of Sporulation Operons

By S. M. TURNER, J. ERRINGTON AND J. MANDELSTAM*

Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road,
Oxford OX1 3QU, UK

(Received 23 May 1986; revised 23 June 1986)

The sporulation gene *spoIIIC* from *Bacillus subtilis* was fused to the *lacZ* gene from *Escherichia coli*, so that the transcription of the *lacZ* gene was under the control of the *spoIIIC* promoter. Production of β -galactosidase, under conditions of sporulation, was then used as an indicator to study the expression of *spoIIIC* in relation to other sporulation loci. Expression of *spoIIIC*, which occurred only in the mother cell compartment, was prevented by mutations in all of the stage 0 and stage II loci, and also by *spoIID*, *spoIVA* and *spoIVB* mutations. By contrast, the last three operons are not needed for expression of *spoVA*, which has previously been shown to be spore-specific. In consequence, a branched pathway of gene expression is proposed. One branch leads to expression of *spoVA* within the spore compartment, the other to expression of *spoIIIC* in the mother cell.

INTRODUCTION

It has been suggested that the expression of sporulation operons could occur in a linear dependent sequence, i.e. a sequence in which each step is dependent on the one before (Mandelstam, 1976). The possibility has also been considered that there might be parallel or branched pathways, but there has been no firm evidence for such an assumption (Coote & Mandelstam, 1973; Piggot & Coote, 1976), although Lencastre & Piggot (1979) obtained indirect genetic evidence that some operons might be expressed specifically in one of the cell compartments (i.e. spore or mother cell).

Investigation of the sequential expression of various sporulation operons has been greatly facilitated by the construction of genetic fusions to the *Escherichia coli lacZ* gene (e.g. Zuber & Losick, 1983; Stephens *et al.*, 1984). The method has been used to study the control of the sporulation operons *spoIID* (Clarke *et al.*, 1986), *spoIIA* (Errington & Mandelstam, 1986*a*) and *spoVA* (Errington & Mandelstam, 1986*b*), and their dependence on the previous expression of other sporulation operons, i.e. their dependence patterns.

This paper describes a study of the expression of the *spoIIIC* locus of *Bacillus subtilis* using a *lacZ* fusion.

METHODS

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

Induction of sporulation. Bacteria were grown at 37 °C in casein hydrolysate (CH) medium, supplemented with the appropriate growth requirements, until mid-exponential phase, and then induced to sporulate in resuspension medium as described by Sterlini & Mandelstam (1969). The time zero for induction of sporulation and the hourly intervals thereafter are referred to as t_0 , t_1 , etc.

Phage ϕ 105 methods. Methods for the preparation of phage lysates and for infection by, and assay of, phage ϕ 105 and its derivatives are described elsewhere (Jenkinson & Mandelstam, 1983; Errington, 1984; Errington, & Mandelstam, 1986*a*).

Table 1. *Plasmids, bacteriophage and bacterial strains*

	Relevant characteristics	Source or reference
Plasmids		
pMR6	<i>bla</i> ⁺ <i>cat</i> ⁺ <i>spoIIIC</i> ⁺	Gift from A. L. Sonenshein
pSGMU32	<i>bla</i> ⁺ <i>lacZ</i> ⁺ <i>cat</i> ⁺	Errington (1986)
Phage		
φ105DI:1t	4.0 kbp deletion of non-essential DNA	Flock (1977)
φ105J42	Tail-less <i>spoIIIC</i> ⁺ transducing phage	This paper
φ105J43	Tail-less phage carrying <i>spoIIIC::lacZ</i> fusion	
φ105J20	Tail-less phage carrying <i>spoVA::lacZ</i> gene fusion	Errington & Mandelstam (1986b)
Strain of <i>E. coli</i>		
JM103	Δ(<i>lac-pro</i>) <i>thi</i> <i>strA</i> <i>supE</i> <i>endA</i> <i>sbcB15</i> <i>hsdR4</i> F' <i>traD36 proAB lacI^qΔM15</i>	Messing <i>et al.</i> (1981)
Strains of <i>B. subtilis</i> †		
168	Spo ⁺ <i>trpC2</i>	Laboratory stock
SG38	Spo ⁺ <i>trpC2</i>	Errington & Mandelstam (1986a)
23.1	<i>spoIVC23 trpC2 rpoB2</i>	E31, Waites <i>et al.</i> (1970)*
513.2	<i>spoVIA513 metB5</i>	Jenkinson (1981)
590	<i>spoIIIF590 lys-1</i>	Lamont & Mandelstam (1984)
610.1	<i>spoVIC610 pheA12</i>	James & Mandelstam (1985)
618	<i>trpC2 spoIIIC::lacZ-cat</i>	This paper

* E31 is the previous designation of this strain.

† All the other Spo⁻ strains that were used, viz. 1.5, 2.12, 17, 25.7, 36, 43.6, 48.7, 55.3, 65, 67, 85.3, 88.7, 89.10, 91, 92.5, 93.2, 131.5, 134, 141, 156, 165.3, 221.1, 224.1, 298.4, 484.2, 485, 486, 488.8, 496.1, 497.1, 498, 516, 517, 520.3, 522.2, 562.5, 612.2, are listed in Table 1 of Errington & Mandelstam (1986a).

Buffers. Ligation buffer was 50 mM-Tris/HCl pH 7.4, 10 mM-MgCl₂, 10 mM-DTT, 1.0 mM-spermidine, 1.0 mM-ATP, bovine serum albumin (0.1 mg ml⁻¹). TE buffer was 10 mM-Tris/HCl, 1 mM-EDTA (pH 7.5). Klenow reaction buffer (final concentration) was 10 mM-Tris/HCl pH 8.0, 5 mM-MgCl₂.

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

Transformation of *B. subtilis*. Recipient cells of *B. subtilis* were made competent and transformed by the method of Anagnostopoulos & Spizizen (1961) as modified by Jenkinson (1983).

Transformations in *E. coli*. The transformation of plasmid pMR6, isolated and kindly provided by M. Rosenkrantz and A. L. Sonenshein (Tufts University Medical School, Boston, Mass., USA), into *E. coli* strain JM103 was done as described in the Amersham booklet *M13 Cloning and Sequencing Handbook*.

Preparation of DNA. Plasmid DNA was extracted from *E. coli* by the alkaline lysis method of Birnboim & Doly (1979) as described by Maniatis *et al.* (1982). Chromosomal DNA from *B. subtilis* was prepared by the method of Ward & Zahler (1973). Phage DNA was prepared as described by Errington (1984).

When necessary, cohesive ends resulting from digestion with restriction endonucleases were blunted by treatment with the Klenow fragment of DNA polymerase I (Amersham). The DNA, Klenow reaction buffer, 4 μl of a solution containing all four deoxyribonucleotide triphosphates (each at 0.5 mM) and 1 unit of Klenow fragment were suspended in a final volume of 30 μl and incubated at room temperature for 10 min. The mixture was then heated to 70 °C for 10 min to inactivate the enzyme.

Construction of a *spoIIIC::lacZ* transcriptional gene fusion. Plasmid pSG32 (Errington, 1986) is a derivative of plasmid pUC18 (Norrander *et al.*, 1983) containing a 4.8 kbp *Bam*HI insert that includes two genes: a hybrid gene consisting of the amino-terminal-coding region and the ribosome binding site from the *spoIIAA* gene of *B. subtilis* fused in frame to the *lacZ* gene of *E. coli*, and a selectable chloramphenicol resistance gene, *cat*. This *lacZ-cat* cartridge was constructed in such a way that the *lacZ* gene can be expressed only if the cartridge is fused to the promoter of an adjacent gene, while the chloramphenicol gene is transcribed constitutively from its own promoter. Plasmid pSG32 (4 μg) was digested first with *Sma*I and then with *Hind*III to excise the *lacZ-cat* cartridge.

Fig. 1. Construction of a *spoIIC::lacZ* fusion. The lowest part of the figure shows a restriction map of the *spoIIC* region of the chromosome, incorporating sites for the following enzymes: *BglII* (B), *ClaI* (C), *EcoRI* (E), *EcoRV* (R), *HindIII* (H), *PvuI* (P) and *PvuII* (V). Below, the horizontal arrow indicates the direction and approximate location of *spoIIC* transcription (A. L. Sonenshein, personal communication). The upper part of the figure shows the molecules which were involved in the construction of the *spoIIC::lacZ* transcriptional fusion. The hatched box represents the *lacZ* gene, which is actually a hybrid gene consisting of part of the upstream non-translated region (including ribosome binding site) and the first 23 codons from the *spoIIAA* gene fused, in frame, to all but the first 8 codons of *lacZ* (Errington, 1986). Unlike the wild-type *lacZ* gene (Donnelly & Sonenshein, 1984), this hybrid gene is efficiently translated in *B. subtilis* (Errington, 1986; Errington & Mandelstam, 1986a). The filled box represents a selectable chloramphenicol resistance determinant, *cat*. This '*lac-cat* cartridge' was released from plasmid pSGMU32 (not shown) by digestion with *SmaI* and *HindIII* and was ligated to fragments of DNA having homology to the *spoIIC* region of the *B. subtilis* chromosome, which were obtained by digestion of plasmid pMR6. The latter fragments, which are shown as thin straight lines, have been aligned over their respective regions of homology with the chromosome. DNA segments of plasmid origin are shown as wavy lines. As indicated by the dotted lines, the *SmaI* end of the *lac-cat* cartridge could ligate only to a pMR6 fragment possessing a *PvuI* end (as indicated by the square brackets, these ends were blunted before ligation). Similarly, the *HindIII* end of the cartridge could ligate only to other *HindIII* ends in the mixture. After transformation of a *B. subtilis* recipient strain, only an arrangement of the type shown for ligation of the participating molecules, and a crossover event in both regions of homology (delimited by broken lines), could give rise to stable chloramphenicol-resistant clones (Niaudet *et al.*, 1985; Errington, 1986). The result of the recombination event shown would be the replacement of a *PvuI-HindIII* fragment of the recipient chromosome by the *lac-cat* cartridge, which would lie in an orientation such that *lacZ* expression could occur from the *spoIIC* promoter. Also possible, but not shown in this figure, would be transformants in which the *lac-cat* cartridge was inserted in the reverse orientation, in place of the smaller *HindIII-PvuI* fragment.

M. Rosenkrantz and A. L. Sonenshein kindly provided us with plasmid pMR6, which was thought to contain the intact *spoIIC* locus as a 4.7 kbp *EcoRI* insertion in plasmid pBR325. The lower part of Fig. 1 shows a restriction map of the insert in plasmid pMR6 and the tentative location and orientation of the *spoIIC* mRNA transcript (W. Finston, S. Rong & A. L. Sonenshein, personal communication).

Plasmid pMR6 (5 µg) was digested with *PvuI*, and the resultant cohesive ends blunted with Klenow fragment and then digested with *HindIII*. A portion (2 µg) of the *lacZ-cat* cartridge from pSG32 was ligated to 2.5 µg plasmid pMR6 DNA, pretreated as described above. The ligation mixture was incubated at 4 °C for 16 h and then used to transform competent cells of *B. subtilis* strain CU267. Transformed bacteria were plated on nutrient agar (Oxoid) containing chloramphenicol (5 µg ml⁻¹) and 4-methylumbelliferyl-β-D-galactoside (MUG; 50 µg ml⁻¹), which yields a fluorescent hydrolysis product with β-galactosidase (Youngman *et al.*, 1985). After overnight incubation at 37 °C colonies were inspected for fluorescence over an ultra-violet transilluminator.

Fluorescent colonies were subcultured on nutrient agar to check that these isolates were Spo⁻ and stable.

Measurement of β-galactosidase activity. Samples (0.5 ml) from sporulating cultures were assayed for β-galactosidase activity using a method based on hydrolysis of MUG (Errington & Mandelstam, 1986a). One unit of β-galactosidase catalyses the hydrolysis of 1 nmol MUG min⁻¹ in the standard assay.

Measurement of alkaline phosphatase activity. Samples of sporulating cell cultures (1.5 ml) were assayed for alkaline phosphatase activity as described by Errington & Mandelstam (1983). One unit of alkaline phosphatase hydrolyses 1 nmol of substrate, *p*-nitrophenyl phosphate, in 15 min at 30 °C.

β -Galactosidase distribution in mother cell and spore fractions. Samples from sporulating cultures at $t_{5.5}$ were divided into 'spore' (pellet) and 'mother cell' (supernatant) fractions after passage through a French pressure cell as described previously (Errington & Mandelstam, 1986b). The resultant 0.5 ml samples were treated with or without germinant as described previously except that the final volume of the treated samples was made to 600 μ l rather than to 1 ml (i.e. a more concentrated solution of germinants was added).

RESULTS

Construction of a $spoIIC::lacZ$ transcriptional fusion

To construct a $spoIIC::lacZ$ transcriptional fusion, we first digested plasmid pMR6 separately with *Hind*III and with *Pvu*I, and then ligated the resulting fragments to a *lac-cat* cartridge (Errington, 1986) in such a way as to provide the cartridge with flanking regions that were homologous to the *B. subtilis* chromosome in the $spoIIC$ region; transformation of the product of the ligation mixture into *B. subtilis* would then lead to insertion of the cartridge into $spoIIC$ (Fig. 1). [The *lac-cat* cartridge was excised from plasmid pSGMU32 by digestion with *Hind*III and *Sma*I. Ligation of *Pvu*I with *Sma*I ends was made possible by 'filling in' the *Pvu*I cohesive ends (see Methods).] The ligation mixture was transformed into the *B. subtilis* wild-type strain 168 with selection for chloramphenicol resistance. This procedure selected for integration of the *lac-cat* cartridge in place of one or other of the *Hind*III-*Pvu*I fragments in the $spoIIC$ region of the recipient chromosome (Niaudet *et al.*, 1985; Errington, 1986). As expected, of about 100 transformants, almost all were stably Spo^- as a result of the chromosomal disruption, and about 15% of these produced β -galactosidase as judged by fluorescence on plates containing the indicator MUG (Youngman *et al.*, 1985), presumably as a result of transcription of the *lacZ* gene from the $spoIIC$ promoter. The non-fluorescent Spo^- colonies presumably contained an integrated *lac-cat* cartridge in the wrong orientation (i.e. the reverse of that shown in Fig. 1). Differences in the sizes of the flanking homologous fragments of DNA probably account for the observed inequality of recovery of the alternative classes.

The 4.7 kbp *Eco*RI fragment of *B. subtilis* DNA in plasmid pMR6 was thought to contain the intact $spoIIC$ transcription unit (see above). To test for complementation of mutation $spoIIC496$ (previously called 94U; Ionesco *et al.*, 1970) and of the new $spoIIC::lacZ-cat$ mutant (designated strain 618), this fragment was subcloned into phage $\phi 105DI::1t$ by prophage transformation (see Methods). A $spoIIC^+$ transducing phage was isolated (designated $\phi 105J42$) and its restriction map is shown in Fig. 2. In this phage, *Eco*RI fragments E (5.3 kbp) and I (0.48 kbp) of $\phi 105DI::1t$ have been replaced by a new fragment of the expected size, 4.7 kbp. As with other phages having a deletion of *Eco*RI fragments in the same region, $\phi 105J42$ is tail-less and can transduce only in the presence of wild-type helper phage (Iijima *et al.*, 1980; Jenkinson & Mandelstam, 1983). Transduction of strains 496.1, carrying the original mutation, $spoIIC496$, and 618, containing the new chromosomal disruption, in both cases led to the production of spores resistant to chloroform vapour.

Transfer of the chromosomal $spoIIC::lacZ$ gene fusion to phage $\phi 105J42$

As described for the analysis of *lacZ* gene fusions to both $spoIIA$ and $spoVA$ in an accompanying paper (Errington, 1986), analysis of expression is facilitated by transfer of the gene fusion to phage $\phi 105$, which can be used to lysogenize strains of differing genotype. To bring about this transfer, chromosomal DNA from strain 618 was used to transform a derivative of strain 496.1, which was lysogenic for phage $\phi 105J42$ and hence phenotypically Spo^+ . Insertion of the transcriptional fusion into the prophage by a double-crossover recombination event would be expected to give rise to Spo^- transformants, whereas integration at the chromosomal $spoIIC$ locus would give transformants that remained Spo^+ (Errington, 1986). Unfortunately, it was not easy to distinguish the difference between Spo^+ and Spo^- transformants on plates containing chloramphenicol, so all the chloramphenicol-resistant transformants were pooled and a phage lysate was prepared, which was then tested for

Fig. 2. *EcoRI* restriction maps of phages $\phi 105DI:lt$, $\phi 105J42$ and $\phi 105J43$. The vertical arrows indicate the locations of *EcoRI* restriction sites. One letter designations for the fragments of phage origin are given (Bugaichuk *et al.*, 1984). Fragment B' was derived from fragment B of wild-type $\phi 105$ by deletion of 4 kbp of non-essential DNA (Flock, 1977). The open box represents chromosomal DNA from the *spoIIIC* region, and the direction and approximate extent of *spoIIIC* transcription (A. L. Sonenshein, personal communication) is indicated by a horizontal arrow. The hatched box and the filled box represent the *lacZ* and chloramphenicol-resistance genes respectively. Broken lines indicate the boundaries of the gene replacement events that occurred during the construction of $\phi 105J42$ and $\phi 105J43$ (see text).

transduction of chloramphenicol-resistance to recipient strain CU267. Phage DNA from a purified transductant was subjected to restriction mapping and the deduced structure of this phage, designated $\phi 105J43$, is shown in Fig. 2. The new phage had a structure similar to that of $\phi 105J42$ except that the 0.8 kbp *PvuI*–*HindIII* fragment of DNA from the *spoIIIC* region was replaced, as expected, by a 4.8 kbp DNA segment corresponding to the *lacZ*–*cat* cartridge. The orientation of the cartridge was such that transcription of the *lacZ* gene was in the proposed direction for *spoIIIC* expression (W. Finston, S. Rong & A. L. Sonenshein, personal communication). In plate tests, all of the chloramphenicol-resistant transductants in this and in subsequent experiments with $\phi 105J43$ in appropriate recipient strains produced colonies that showed β -galactosidase activity.

*Production of β -galactosidase from the *spoIIIC* : *lacZ* fusion under sporulation conditions*

A *Spo*⁺ strain of *B. subtilis*, SG38, was infected with $\phi 105J43$ and used as a positive control for all sporulation experiments. The activity of β -galactosidase was measured in this strain at t_0 , and at intervals thereafter. The results of a typical time course are shown in Fig. 3. β -Galactosidase began to be produced at approximately $t_{2.5}$ and reached a maximum at t_5 . A similar pattern was observed when strain 618 (the strain carrying the *spoIIIC* : *lacZ* gene fusion on the chromosome) was transferred to sporulation medium. This was also found with all the sporulation mutants infected with $\phi 105J43$ that did not prevent expression of the *spoIIIC* : *lacZ* gene fusion (see below), with the exception of strains harbouring mutations *spoIIIF590* and *spoVIC610*. In such strains, expression of β -galactosidase was delayed by 1 h and it continued to increase throughout the experiment (Fig. 3). The production of alkaline phosphatase, a biochemical marker event which normally occurs at about t_1 , was also delayed by 1 h in these strains as was the occurrence of later events.

Although *B. subtilis* does not grow on lactose, it has a β -galactosidase-like activity that gives rise to a background of fluorescence under standard assay conditions (see Errington & Mandelstam, 1986*a*). The results for *B. subtilis* strain 168, the *Spo*⁺ wild-type carrying no *lacZ* fusion, are therefore included in Fig. 3. The background activity was low and there was no difficulty in distinguishing strains that produced β -galactosidase from those that did not.

Fig. 3. Measurement of β -galactosidase activity in sporulating cells of strain 168 (Spo^+ , no *lacZ* fusion, ○), and of the following strains lysogenic for $\phi 105J43$ (*spoIIC::lacZ*): SG38 (Spo^+ , ●), 610.1 (*spoVIC610*, □) and 590 (*spoIIIF590*, ■).

Table 2. *Effect of various sporulation mutations on the expression of spoIIC*

The *spoIIC::lacZ* gene fusion was transferred to each of the sporulation mutants by infection with phage $\phi 105J43$ and subsequent selection for chloramphenicol resistance. Cells were induced to sporulate and samples were taken at 1 h or 0.5 h intervals, for β -galactosidase assay. Each strain was tested for β -galactosidase production on at least two separate occasions. +, Activity reaching 55% or more of wild-type activity; +*, activity reaching 55% or more of wild-type activity but delayed by about 1 h; -, the rapid increase in activity observed at about $t_{2.5}$ in cultures giving positive results was absent, and the total activity was always less than 30% of that seen in the wild-type.

Mutated locus	β -Galactosidase activity						<i>Ger</i>
	0-1	II	III	IV	V	VI	
<i>A</i>	-	-	+	-	+	+	
<i>B</i>	-	-	+	-	+	+	
<i>C</i>	-	-	+	+	+	+	
<i>D</i>	-	-	-	+	+		
<i>E</i>	-	-	+	+	+		+
<i>F</i>	-	-	+	+	+		
<i>G</i>	-	-					
<i>H</i>	-	-			+		
<i>J</i>	-	-			+		
<i>K</i>	-	-					

Effect of various sporulation mutations on the expression of spoIIC::lacZ

Each of the sporulation mutant strains listed in Table 1 was infected with $\phi 105J43$, a phage carrying the *spoIIC::lacZ* fusion. Although the *spoIIC* gene on this phage was disrupted by the *lac-cat* insertion, each sporulation mutant (except strain 496.1) carried a functional copy of *spoIIC* in its chromosome. The effect of each *spo* mutation on the expression of *spoIIC* was determined by measurement of β -galactosidase at intervals after the induction of sporulation (Table 2). All of the mutations that blocked sporulation at stage 0 and at stage II also blocked expression of *spoIIC*, whereas those affecting stage V or stage VI, and a *gerE* mutation, did not. Mutations in *spoVIC* and *spoIIIF* caused a reproducible delay in its expression of about 1 h (see above). Mutations in *spoIIID*, *spoIVA* and *spoIVB* blocked expression of the *spoIIC* gene, but those in the other stage III and stage IV loci did not.

Table 3. Location of β -galactosidase activity in sporulating cells of strain SG38 ($\phi 105J43$)

Strain SG38 ($\phi 105J43$) was induced to sporulate and at $t_{5.5}$ the cells were harvested. Various fractions were assayed for β -galactosidase after incubation with or without germinants (see Methods).

Sample	β -Galactosidase activity (units ml ⁻¹)*	
	Ungerminated	Germinated
Whole culture	0.24	0.22
Whole culture after cell breakage (French pressure cell)	0.22	0.22
'Mother cell fraction' (supernatant)	0.22	0.22
'Spore fraction' (pellet)	0.019	0.022

* Cell samples from 25 ml of sporulating culture were resuspended in 5 ml of sodium phosphate buffer. All values given are for these concentrated suspensions and should be divided by five for direct comparison with other values from β -galactosidase assays.

Location of β -galactosidase activity in differentiating sporulating cells

We have previously shown (Errington & Mandelstam, 1986*b*) that expression of the *spoVA* operon occurs predominantly, perhaps exclusively, in the spore compartment. To determine in which of the cell compartments *spoIIC* expression occurs, we fractionated the differentiating cells (see Methods) and measured β -galactosidase activity in the spore and mother-cell fractions.

Derivatives of strain SG38 (*Spo*⁺) lysogenic for phages $\phi 105J20$ (*spoVAA::lacZ*) or $\phi 105J43$ (*spoIIC::lacZ*) were induced to sporulate, and at $t_{5.5}$ the cells were harvested, divided into spore and mother-cell fractions, and assayed for β -galactosidase both before and after treatment with spore germinants (see Methods). The results (Table 3) clearly show that about 90% of the β -galactosidase produced from the *spoIIC::lacZ* fusion gene resides in the mother-cell compartment. Germination had no effect on the observed activities. This contrasts with the observation that β -galactosidase activity expressed from a *spoVAA::lacZ* gene fusion was located predominantly in the spore compartment (Errington & Mandelstam, 1986*b*). Full detection of this activity was only possible after treatment of the developing spores with germinants which sensitizes them to toluene and makes them permeable to the β -galactosidase substrate MUG (Errington & Mandelstam, 1986*b*).

These results clearly support the notion that *spoIIC* expression and *spoVA* expression occur in different cell compartments.

DISCUSSION

In the wild-type and in most of the mutants in which *spoIIC* is expressed, the appearance of β -galactosidase indicates that the operon is 'turned on' at about $t_{2.5}$ (Fig. 3). It is thus expressed at about the same time as *spoVA* (Errington & Mandelstam, 1986*b*), and about 1 to 1.5 h after *spoIIA* (Errington & Mandelstam, 1986*a*) and *spoIID* (Clarke *et al.*, 1986). The delayed expression of sporulation events in some mutants, e.g. *spoIIIF* and *spoVIC*, has been noted before (James & Mandelstam, 1985; Errington & Mandelstam, 1986*a*) and we have no explanation for it.

The expression of *spoIIC* is dependent on the expression of all the known stage 0 operons and of all those that are known to control stage II (Table 2). Moreover, its expression is prevented by mutations in *spoIIID*, *spoIVA* and *spoIVB*. In Table 4 we have compared the dependence pattern of this operon with those of *spoIIA*, *spoIID* and *spoVA*, which have all been examined by the use of the appropriate *lacZ* fusions. The contrast in the dependence patterns of *spoIIC* and *spoVA* will be apparent. Both operons depend on the correct expression of all the operons involved in sporulation up to the end of stage II. The line of dependence then branches: expression of *spoIIC* depends on *spoIIID*, *spoIVA* and *spoIVB* and is independent of *spoIIIA*, *spoIIIB* and *spoIIIE* (see below). The converse pattern of dependence holds for *spoVA*.

Table 4. *Summary of the effects of mutations at spo loci on expression of various spo :: lacZ fusions*

Results for the four fusions are taken from the following sources: *spoIIA::lacZ*, Errington & Mandelstam (1986*a*); *spoIID::lacZ*, Clarke *et al.* (1986); *spoIIIC::lacZ*, this paper; *spoVA::lacZ*, Errington & Mandelstam (1986*b*). -, Prevents expression of *spo::lacZ*; +, allows expression of *spo::lacZ*.

<i>spo</i> mutant background	Expression of fusion			
	<i>spoIIA::lacZ</i>	<i>spoIID::lacZ</i>	<i>spoIIIC::lacZ</i>	<i>spoVA::lacZ</i>
0A, 0B, 0D, 0E, 0F, 0G, 0H	-	-	-	-
IIA, IIE, IIG	+	-	-	-
IIB, IID	+	+	-	-
IIID, IVA, IVB	+	+	-	+
IIIA, IIIB, IIIE	+	+	+	-
All others tested	+	+	+	+

If these conclusions are combined with those derived earlier from the dependence patterns of operons up to the end of stage II (Clarke *et al.*, 1986), we arrive at the scheme shown below, in which operons whose order of expression is still undetermined are bracketed together (asterisks indicate that the dependence pattern was determined by the use of *lacZ* fusions). The dependence of expression of *spoIIG* upon that of *spoIIA* was determined by immunological methods (Trempey *et al.*, 1985). It thus appears that the single linear dependence sequence (see Mandelstam, 1976) holds until the end of stage II; it then becomes complicated by branching into two subsidiary sequences each of which also seems to exhibit dependence.

From the data in Table 3 it appears that *spoIIIC* is expressed in the mother cell. The small amount of β -galactosidase activity found in the spore fraction is not increased by treatment with germinants and this probably means that it is the result of adsorption of a small amount of the enzymes to the spores. This contrasts with the results found with *spoVA* which indicated that the operon is expressed only in the spore compartment: virtually all of the β -galactosidase activity was found in the spore fraction and its detection was greatly enhanced by treatment with germinants, presumably as a result of changes in spore permeability (Errington & Mandelstam, 1986*b*). (Although the results obtained for the two different *spo::lacZ* fusions help to reinforce each other, we hope to confirm the cellular location of β -galactosidase in these strains by immuno-electron microscopy.) It is also consistent with the above scheme that mutations in the *spoIIIC* locus block late sporulation events such as spore coat protein synthesis that presumably occur specifically in the mother cell compartment (unpublished results based on electron microscopy). By contrast, mutations in the *spoVA* locus do not block such events (Errington & Mandelstam, 1986*b*). We can therefore surmise that the alternative dependence pathways leading to expression of *spoIIIC* and *spoVA* reflect separate lines of gene expression that occur in the mother cell and spore compartments of the differentiating cell.

We are grateful to Dr A. L. Sonenshein and coworkers for providing us with plasmid pMR6 and with much useful unpublished data. Bridget Harris played an important part in the construction of phage ϕ 105J43 and in characterizing the *spoIIIC::lacZ* fusion. The work was supported by the Science and Engineering Research Council. J.E. is in receipt of a 1983 Research Fellowship of the Royal Society.

REFERENCES

- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology* **81**, 741–746.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- BUGAICHUK, U. D., DEADMAN, M., ERRINGTON, J. & SAVVA, D. (1984). Restriction enzyme analysis of *Bacillus subtilis* bacteriophage ϕ 105 DNA. *Journal of General Microbiology* **130**, 2165–2167.
- CLARKE, S., LOPEZ-DIAZ, I. & MANDELSTAM, J. (1986). Use of *lacZ* gene fusions to determine the dependence pattern of the sporulation gene *spoIID* in *spo* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2987–2994.
- COOTE, J. G. & MANDELSTAM, J. (1973). Use of constructed double mutants for determining the temporal order of expression of sporulation genes in *Bacillus subtilis*. *Journal of Bacteriology* **114**, 1254–1263.
- DONNELLY, C. E. & SONENSHEIN, A. L. (1984). Promoter probe plasmid for *Bacillus subtilis*. *Journal of Bacteriology* **157**, 965–967.
- ERRINGTON, J. (1984). Efficient *Bacillus subtilis* cloning system using bacteriophage vector ϕ 105J9. *Journal of General Microbiology* **130**, 2615–2628.
- ERRINGTON, J. (1986). A general method for fusion of the *Escherichia coli lacZ* gene to chromosomal genes in *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2953–2966.
- ERRINGTON, J. & MANDELSTAM, J. (1983). Variety of sporulation phenotypes resulting from mutations in a single regulatory locus, *spoIIA*, in *Bacillus subtilis*. *Journal of General Microbiology* **129**, 2091–2101.
- ERRINGTON, J. & MANDELSTAM, J. (1986a). Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIA* in *spo* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2967–2976.
- ERRINGTON, J. & MANDELSTAM, J. (1986b). Use of a *lacZ* gene fusion to determine the dependence pattern and the spore compartment expression of sporulation operon *spoVA* in *spo* mutants of *Bacillus subtilis*. *Journal of General Microbiology* **132**, 2977–2985.
- FLOCK, J.-I. (1977). Deletion mutants of temperate *Bacillus subtilis* bacteriophage ϕ 105. *Molecular and General Genetics* **155**, 241–247.
- IJIMA, T., KAWAMURA, F., SAITO, H. & IKEDA, Y. (1980). A specialized transducing phage constructed from *Bacillus subtilis* phage ϕ 105. *Gene* **9**, 115–126.
- IONESCO, H., MICHEL, J., CAMI, B. & SCHAEFFER, P. (1970). Genetics of sporulation in *Bacillus subtilis* Marburg. *Journal of Applied Bacteriology* **33**, 13–24.
- JAMES, W. & MANDELSTAM, J. (1985). *spoVIC*, a new sporulation locus in *Bacillus subtilis* affecting spore coats, germination and the rate of sporulation. *Journal of General Microbiology* **131**, 2409–2419.
- JENKINSON, H. F. (1981). Germination and resistance defects in spores of a *Bacillus subtilis* mutant lacking a coat polypeptide. *Journal of General Microbiology* **127**, 81–89.
- JENKINSON, H. F. (1983). Altered arrangement of proteins in the spore coat of a germination mutant of *Bacillus subtilis*. *Journal of General Microbiology* **129**, 1945–1958.
- JENKINSON, H. F. & MANDELSTAM, J. (1983). Cloning of the *Bacillus subtilis* *lys* and *spoIIIB* genes in phage ϕ 105. *Journal of General Microbiology* **129**, 2229–2240.
- LAMONT, I. L. & MANDELSTAM, J. (1984). Identification of a new sporulation locus, *spoIIIF*, in *Bacillus subtilis*. *Journal of General Microbiology* **130**, 1253–1261.
- LENCASTRE, H. DE & PIGGOT, P. J. (1979). Identification of different sites of expression for *spo* loci by transformation of *Bacillus subtilis*. *Journal of General Microbiology* **114**, 377–389.
- MANDELSTAM, J. (1976). Bacterial sporulation: a problem in the biochemistry and genetics of a primitive developmental system. *Proceedings of the Royal Society B* **193**, 89–106.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MESSING, J., CREA, R. & SEEBURG, P. H. (1981). A system for shotgun DNA sequencing. *Nucleic Acids Research* **9**, 309–321.
- MOIR, A. (1981). Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. *Journal of Bacteriology* **146**, 1106–1116.
- NIAUDET, B., JANNIERE, L. & EHRLICH, S. D. (1985). Integration of linear, heterologous DNA molecules into the *Bacillus subtilis* chromosome: mechanism and use in induction of predictable rearrangements. *Journal of Bacteriology* **163**, 111–120.
- NORRANDER, J., KEMPE, T. & MESSING, J. (1983). Improved M13 vectors using oligonucleotide-directed mutagenesis. *Gene* **26**, 101–106.
- PIGGOT, P. J. & COOTE, J. G. (1976). Genetic aspects of bacterial endospore formation. *Bacteriological Reviews* **40**, 908–962.
- STEPHENS, M. A., LANG, M., SANDMAN, K. & LOSICK, R. (1984). A promoter whose utilization is temporally regulated during sporulation in *Bacillus subtilis*. *Journal of Molecular Biology* **176**, 333–348.
- STERLINI, J. M. & MANDELSTAM, J. (1969). Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. *Biochemical Journal* **113**, 29–37.
- TREMPY, J. E., MORRISON-PLUMMER, J. & HALDENWANG, W. G. (1985). Synthesis of σ^{29} , an RNA polymerase specificity determinant, is a developmentally regulated event in *Bacillus subtilis*. *Journal of Bacteriology* **161**, 340–346.
- WAITES, W. M., KAY, D., DAWES, I. W., WOOD, D. A., WARREN, S. C. & MANDELSTAM, J. (1970). Sporulation in *Bacillus subtilis*. Correlation of biochemical events with morphological changes in asporogenous mutants. *Biochemical Journal* **118**, 667–676.
- WARD, J. B., JR & ZAHLER, S. A. (1973). Genetic studies of leucine biosynthesis in *Bacillus subtilis*. *Journal of Bacteriology* **116**, 719–726.
- YOUNGMAN, P., ZUBER, P., PERKINS, J. B., SANDMAN, K., IGO, M. & LOSICK, R. (1985). New ways to study developmental genes in spore-forming bacteria. *Science* **228**, 285–291.
- ZUBER, P. & LOSICK, R. (1983). Use of a *lacZ* fusion to study the role of the *spo* genes of *Bacillus subtilis* in developmental regulation. *Cell* **35**, 275–283.