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*

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A *spoVAA::lacZ* gene fusion has been used to study expression of the *spoVA* operon during sporulation in *Bacillus subtilis*. β -Galactosidase activity, encoded by the fusion gene, begins to be produced about 2.5 h after the induction of sporulation, well before the phenotypic consequences of *spoVA* mutations are manifested. *spoVA* expression is dependent on all of the known *spo0* and *spoII* loci and on some of the 'early' *spoIII* loci, but not on 'later' loci. Several lines of evidence suggest that *spoVA* expression occurs only in the spore compartment. The implications of this observation for models of the overall regulation of gene expression during sporulation are discussed.

INTRODUCTION

The sporulation operon *spoVA* is one of more than 50 known genetic loci specifically concerned with the regulation of sporulation in *Bacillus subtilis* (Hranueli *et al.*, 1974; Piggot & Coote, 1976; Piggot *et al.*, 1981). In addition to the single mutation that first defined the locus (89; Hranueli *et al.*, 1974), seven further *spoVA* mutations have been isolated following directed mutagenesis of the *lys-1* region of the *B. subtilis* chromosome (Errington & Mandelstam, 1984). The locus has been cloned (Savva & Mandelstam, 1984; Errington, 1984) and sequenced (Fort & Errington, 1985) and the sequence indicates the existence of at least five genes, designated *A*, *B*, *C*, *D* and *E*, which are transcribed as a single polycistronic mRNA (Fort & Errington, 1985; Savva & Mandelstam, 1985, 1986). An extensive region of homology between genes *C* and *E* suggests that one of them may have arisen by gene duplication (Errington *et al.*, 1985). Complementation analysis of eight *spoVA* mutations indicates that they fall into four different genes. A mutation in any one of these genes causes sporulation to be blocked at a late stage. *spoVA* mutants synthesize alkaline phosphatase and glucose dehydrogenase (stage II and stage III marker events respectively) but not dipicolinic acid, which normally appears during stage V. Although the phase-grey spores which they produce are sensitive to heat and to chloroform, they are partially resistant to toluene and to lysozyme (Errington & Mandelstam, 1984). Lencastre & Piggot (1979) obtained genetic evidence indicating that the *spoVA* locus might be expressed in the spore compartment of the sporulating cell. Other groups have also suggested that certain gene products have a differential compartmental distribution (Andreoli *et al.*, 1975; Nakayama *et al.*, 1981). These findings raise the possibility that during sporulation there may be independent sequences of gene expression in the spore and in the mother-cell; these cells have different developmental fates and are separated initially by a double membrane and later by additional cortical and coat layers.

This paper describes the use of a *spoVA::lacZ* gene fusion, constructed as described in an accompanying paper (Errington, 1986), to analyse expression of the *spoVA* locus during sporulation. An analysis of the effects of mutations at other *spo* loci has been used to investigate

Table 1. *Bacillus subtilis* strains

Strain	Genotype*	Origin
614 (Spo ⁻)	<i>lys-1 metC3 tal-1 spoVAA::lacZ-cat</i>	Errington (1986)
SG37 (Spo ⁺)	<i>lys-1 metC3 tal-1 (φ105J20</i>	
	<i>spoIIA⁺ spoVAA::lacZ-cat)</i>	
614.1 (Spo ⁻)	<i>trpC2 spoVAA::lacZ-cat</i>	Transformation of strain SG38 with DNA from strain 614 and selection for Cm ^R
89.9	<i>spoVAE89 lys-1 tal-1</i>	Errington & Mandelstam (1984)

* *tal-1*, resistance to β -thienylalanine; *cat*, resistance to chloramphenicol.

the genetic control of the *spoVA* locus, and accompanying papers deal with similar analyses of the *spoIIA* locus (Errington & Mandelstam, 1986) and the *spoIID* locus (Clarke *et al.*, 1986).

METHODS

Bacterial strains. These are listed in Table 1, except for the Spo⁺ and Spo⁻ strains listed in Table 1 of the preceding paper (Errington & Mandelstam, 1986).

General methods. Media, techniques for growth and sporulation, transformation, transduction and β -galactosidase assays using 4-methylumbelliferyl- β -D-galactopyranoside (MUG) are all detailed in the preceding paper (Errington & Mandelstam, 1986). One unit of β -galactosidase catalyses the production of 1 nmol 4-methylumbelliferone min⁻¹ at 30 °C using the standard assay described.

***spoVAA::lacZ* gene fusion.** An accompanying paper (Errington, 1986) describes the construction of a chromosomal *spoVAA::lacZ* fusion and a transducing phage ϕ 105J20. The latter can be used to transfer the *spoVA* regulatory sequences and gene fusion to other bacterial strains by transduction followed by selection for chloramphenicol resistance. The structure of the DNA insert in this phage is shown in Fig. 1. The hybrid gene consists of the first 35 codons of the *spoVAA* gene, followed by an eight codon spacer region of 'synthetic' origin, and then all but the first eight codons of the *lacZ* gene (Kalinins *et al.*, 1983).

β -Galactosidase distribution in mother cell and forespore fractions. Samples (50 ml) of sporulating cultures were harvested by centrifugation, washed in ice-cold Tris/HCl buffer (50 mM, pH 7.5) and frozen at -70 °C. The thawed pellet was resuspended in 10 ml sodium phosphate buffer (0.1 M, pH 7.0). A 4 ml portion of the cell suspension was passed twice through a French pressure cell at 83 MPa, which released >90% of the spores from their mother cells (Jenkinson *et al.*, 1981). All but 1 ml of the suspension was then centrifuged at low speed (800 g, 4 min) to harvest the spores, and the supernatant ('mother cell' fraction) was carefully decanted. The spores were washed and resuspended in 3 ml sodium phosphate buffer. Samples (1 ml) of the whole cell suspension, both before and after French press treatment, and of the 'spore' and 'mother cell' fractions were then divided into two 0.5 ml portions. To one portion of each type was added 0.5 ml sodium phosphate buffer ('- germinant'); to the other ('+ germinant') was added 0.5 ml sodium phosphate buffer containing L-alanine (20 mM), KCl (60 mM), D-glucose (20 mM) and D-fructose (20 mM). The spore suspensions were incubated at 37 °C for 1 h to allow germination to occur and β -galactosidase was determined by the MUG assay (Errington & Mandelstam, 1986).

Preparation of spore coat proteins. Preparation of spores and of spore coat fractions, and extraction of spore coat proteins have been described in detail by Jenkinson *et al.* (1981).

SDS-PAGE. Spore coat proteins were electrophoresed on 1.5 mm thick polyacrylamide slab gels (15%, w/v) using the discontinuous buffer system of Laemmli & Favre (1973) as described by Hames (1981). Protein bands were then stained with Coomassie brilliant blue R250 as described by Jenkinson *et al.* (1981). Molecular mass reference proteins (Pharmacia) were as follows: phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α -lactalbumin (14400).

RESULTS

Time of expression of spoVAA::lacZ during sporulation

The time course for β -galactosidase synthesis under sporulation conditions was similar in strains carrying the gene fusion in the chromosomal location (strain 614; phenotypically SpoVA⁻) or on the ϕ 105J20 prophage (strain SG37; phenotypically SpoVA⁺). Activity began to appear at about $t_{2.5}$, reached a peak at t_4 or t_5 and then declined towards the background level (Fig. 2). The amount of activity in a strain, MB75, with no *lacZ* fusion was insignificant.

Fig. 2

Fig. 3

Fig. 2. β -Galactosidase production during sporulation by strains carrying a *spoVAA::lacZ* gene fusion. Strains MB75 (□), 614 (○) and SG37 (●) were induced to sporulate and at intervals samples were taken and assayed for β -galactosidase as described in Methods.

Fig. 3. β -Galactosidase production during sporulation in strains carrying a *spoVAA::lacZ* gene fusion on the ϕ 105J20 prophage. Strains lysogenic for ϕ 105J20 and carrying no *spo* mutation (○) or mutations in the *spoIIIC* (□), *spoIIIF* (■) or *spoVB* (▲) loci were induced to sporulate as described in Methods. At intervals, samples were taken and assayed for β -galactosidase activity.

Effects of *spo* mutations on *spoVAA::lacZ* expression

The *spoVAA::lacZ* fusion, carried on the ϕ 105J20 prophage, was transferred to each member of the series of *spo* mutant strains listed in Table 1 of the preceding paper (Errington & Mandelstam, 1986), by selection for transduction of the phage-mediated chloramphenicol resistance. Each of the derivative strains was induced to sporulate, and samples were taken for assay of β -galactosidase at t_0 and t_4 . The results (means of at least two independent experiments) are summarized in Table 2. Strains with mutations in stage 0 and stage II loci all showed negligible β -galactosidase activities (<7% of the activity of the *Spo*⁺ strain), with the exception of the *spoIIB* mutant, which had an activity somewhat greater than the basal level. The stage III loci were divided into two groups: (a) *spoIIIA*, *spoIIIB* and *spoIIIE* mutants showed negligible β -galactosidase activities (<7% of the activity of the *Spo*⁺ strain); (b) *spoIIIC*, *spoIIID* and *spoIIIF* mutants made appreciable amounts of the enzyme (>19% of the activity of the *Spo*⁺ strain). None of the mutations in 'later' loci prevented synthesis of appreciable amounts of β -galactosidase. Strains with mutations in the *spoIVC*, *spoIVD* and particularly the *spoVE* loci seemed to over-express β -galactosidase in comparison with the *Spo*⁺ control strain.

Since phage ϕ 105J20 carries, in addition to the *spoVAA::lacZ* fusion, an intact, wild-type copy of the *spoIIA* locus, it was not possible to test the effects of *spoIIA* mutations using this system. Instead, DNA from a strain carrying a chromosomal *spoVAA::lacZ* gene fusion was transferred into recipient strains carrying mutations in *spoIIA*. Chloramphenicol-resistant transformants possessing the characteristic *SpoIIA*⁻ phenotype were used for β -galactosidase assays.

In the preceding paper (Errington & Mandelstam, 1986) it is shown that in strains carrying mutations in the *spo0J*, *spoIIIF* and *spoVIC* loci there is delayed expression of the *spoIIAA::lacZ* gene fusion. More detailed time course studies for β -galactosidase expression from the

Table 2. *Effects of spo mutations on the production of β -galactosidase by the *spoVAA::lacZ* gene fusion during sporulation*

Phage ϕ 105J20, carrying a *spoVAA::lacZ* gene fusion, was transduced into a series of *spo* mutant strains (see Table 1 of accompanying paper, Errington & Mandelstam, 1986). Each transductant in turn was induced to sporulate and samples were taken for assay of β -galactosidase. Results shown are the mean activities (as percentages of the wild-type activity) at t_4 , with the number of independent determinations in parentheses. The wild-type Spo⁺ strain containing a *spoVAA::lacZ* fusion (SG37) produced 0.80 units β -galactosidase (ml culture)⁻¹ at t_4 (mean of five determinations). An isogenic strain (MB75) containing no *lacZ* fusion produced 4.1% of the wild-type activity in a control experiment.

Mutated locus	β -Galactosidase activity (% of wild-type activity)						<i>ger</i>
	0	II	III	IV	V	VI	
<i>A</i>	2.6 (2)	4.9 (2)* 3.5 (2)†	2.5 (2)	53 (3)	83 (2)	84 (4)	
<i>B</i>	2.8 (2)	15 (3)	3.1 (2)	31 (3)	80 (4)	79 (3)	
<i>C</i>			19 (6)	160 (4)	110 (2)	31 (2)	
<i>D</i>	4.0 (2)	3.5 (3)	65 (2)	150 (3)	50 (5)		
<i>E</i>	5.0 (2)	6.3 (2)	6.0 (3)	100 (2)	300 (2)		98 (2)
<i>F</i>	5.6 (2)		31 (8)	79 (4)	75 (2)		
<i>G</i>	5.0 (2)	6.1 (3)		58 (3)			
<i>H</i>	4.3 (2)				50 (2)		
<i>J</i>	6.0 (2)				51 (2)		
<i>K</i>	3.4 (2)						

* Result for *spoIIAA* mutation; determined in a strain carrying a chromosomal *spoVAA::lacZ* fusion (see Results).

† Result for *spoIIAC* mutation; determined in a strain carrying a chromosomal *spoVAA::lacZ* fusion (see Results).

spoVAA::lacZ gene fusion in the presence of these and other mutations were done to determine whether similar delays in expression could be identified. Activity was not detected in a *spo0J* mutant but a delay in expression of approximately 1 h was found in strains carrying mutations in the *spoIIIC*, *spoIIIF*, *spoVB* and *spoVIC* loci. The times of expression in some of these mutant strains are shown in Fig. 3.

Forespore-specific expression of the spoVAA::lacZ fusion

β -Galactosidase production in strain SG37 peaks at about t_4 (Fig. 2). On the assumption that *spoVA* is expressed in the spore compartment (see Introduction), one could explain the apparent fall in activity after t_4 by the onset of toluene-resistance that occurs at this time (Jenkinson *et al.*, 1980). Since the β -galactosidase assay depends upon toluene permeabilization of the cell, the development of toluene-resistance in the spore would lead to an apparent fall in enzyme activity caused by failure of the substrate to enter the spore compartment. If this were so, germination, which restores toluene-sensitivity, ought to effect an increase in the measurable activity. Accordingly, strain SG37, a Spo⁺ strain carrying the *spoVAA::lacZ* fusion on the ϕ 105J20 prophage, was induced to sporulate and samples were taken at t_6 and at t_{22} . The cells were harvested, and portions were separated into spore and mother cell fractions, each of which was then assayed for β -galactosidase both before and after treatment with germinants (see Methods). The frequency of germination was much lower in the t_{22} spores than in the t_6 spores. This was understandable because cold-shocked spores at t_6 respond well to germinants (Dion & Mandelstam, 1980), whereas more mature spores germinate efficiently only after heat shock (Powell & Hunter, 1955). Heat-shock treatment was avoided since this might have caused inactivation of β -galactosidase. Nevertheless, treatment of both t_6 and t_{22} spores with germinants led to substantial increases in β -galactosidase activity in all except the mother cell fractions (Table 3). Furthermore, most (70–80%) of the β -galactosidase activity in the germinated samples was found in the spore fractions. These results strongly suggest that most of

Table 3. *Distribution of β -galactosidase activities in the forespore and mother cell fractions of sporulating cells containing a *spoVAA::lacZ* gene fusion*

Strain SG37 was induced to sporulate and samples were harvested at t_6 and at t_{22} . Various fractions were assayed β -galactosidase after incubation with (+) or without (–) germinant (see Methods). The percentage germination in the 'whole culture' samples, as determined by microscopic examination after treatment, was as follows: t_6 with germinant, 57% (74/124); t_6 without germinant, 5% (6/117); t_{22} with germinant, 28% (39/119); t_{22} without germinant, 5% (5/103).

Fraction	Germinant added	β -Galactosidase activity (arbitrary units)	
		t_6	t_{22}
Whole culture	+	1.9	0.51
	–	1.0	0.14
Whole culture after cell breakage (French pressure cell)	+	2.1	0.95
	–	1.5	0.20
'Mother cell fraction' (supernatant)	+	0.36	0.082
	–	0.26	0.095
'Spore fraction' (pellet)	+	1.1	0.31
	–	0.81	0.061

the enzyme activity is in the spore compartment and that its detection by the standard assay is attenuated during development of the spore.

*Synthesis of spore coat proteins by *spoVA* mutants*

Since expression of the *spoVAA::lacZ* fusion gene begins relatively early in sporulation (about $t_{2.5}$) and may be presumed to be spore specific, it was of interest to investigate the effect of *spoVA* mutations on later events that might be considered specific for spore development. Some, at least, of the spore coat proteins are likely to be synthesized specifically in the mother cell, since they form the outer layer of the mature spore (Jenkinson *et al.*, 1981). The structure of the spore coat in *spoVA* mutants might therefore be indicative of the requirement for *spoVA* expression in the mother cell. Spore coat deposition in *spoVA* mutants appears normal as judged by electron microscopy (Piggot & Coote, 1976). To investigate this further, strain 89.9, a *spoVA* mutant, and a *Spo*⁺ strain, 168, were induced to sporulate. At t_9 the spores were harvested and the spore coat proteins were isolated and separated by SDS-PAGE. All of the major coat polypeptides described by Jenkinson *et al.* (1981) were present in both preparations (Fig. 4).

DISCUSSION

Expression of the *spoVAA::lacZ* fusion gene begins at about $t_{2.5}$ (Fig. 2), almost 2 h earlier than the phenotypic lesion of *spoVA* mutations is first manifested through the characteristic absence of dipicolinic acid (Errington & Mandelstam, 1984). The product of the other well-characterized 'stage V' locus, *spoVG*, is expressed even earlier than *spoVA*, viz. within the first hour of sporulation (Rosenbluh *et al.*, 1981). This further emphasizes the notion that the time at which a *spo* gene is expressed may precede by several hours the time at which the phenotype produced by mutations in the gene becomes evident (Wood, 1972; Jenkinson *et al.*, 1980).

Expression of *spoVA* was blocked by mutations in all of the *spo0* and *spoII* loci, and in some of the stage III loci (Table 2). Unexpectedly, mutations in *spoIIIC*, *spoIIID* and *spoIIIF* did not prevent *spoVAA::lacZ* expression, although *spoIIIC* and *spoIIIF* mutations caused a delay of about 1 h in the appearance of β -galactosidase (Fig. 3). However, the phenotypic properties of these mutants, such as the appearance of clearly visible phase-grey spores, suggested that they might be more properly classified as stage IV mutants, as has been suggested previously for the mutation in the *spoIIIC* locus (Piggot & Coote, 1976). The possible mis-classification of *spoIIIF590* may be due to the delay in sporulation that it causes and that is evident both in the expression of *spoVAA::lacZ* and in the stage II *lacZ* gene fusions described in accompanying papers (Errington & Mandelstam, 1986; Clarke *et al.*, 1986). A corresponding delay of induction

Fig. 4. SDS-PAGE patterns (stained with Coomassie blue) of spore coat proteins extracted from t_9 spores of strains 168 (track 2) and 89-9 (track 3). The position of '36K', the major late coat polypeptide of 36 kDa, is indicated. Track 1 contains standard proteins of known size.

could be responsible for the low glucose dehydrogenase activity at t_5 , which formed part of the justification for placing *spo-590* in a new locus, *spoIIIF* (Lamont & Mandelstam, 1984).

Mutations in several other loci also caused a delay in *spoVAA::lacZ* expression. In one of these loci, *spoVIC*, mutations also delayed *spoIIAA::lacZ* expression (Errington & Mandelstam, 1986) and have previously been shown to cause a general delay in sporulation-associated events from an early stage (James & Mandelstam, 1985). Of the other mutants, *spoIIIC496* delayed expression of *spoIID::lacZ* but not that of *spoIIA::lacZ*, while *spoVB* delayed only *spoVAA::lacZ* expression. Thus, it seems that delay in the expression of sporulation specific genes may be relatively common in *spo* mutant strains. Mutation *spoIIIC496*, however, is an extensive chromosomal deletion (> 5 kbp; A. L. Sonenshein, personal communication) that may encompass, in addition to an essential *spo* gene, several other genes of unknown function, the deletion of which might have secondary effects on sporulation.

Lencastre & Piggot (1979) suggested on genetic grounds that the *spoVA* operon needs to be expressed in the forespore compartment for normal development to occur. Here we have presented more direct evidence that this compartment is in fact the site of expression of *spoVA*, but the results do not exclude the possibility that β -galactosidase is not detected in the mother-cell because of rapid turnover. However, the identification of a *spo* locus (*spoIIIC*) that is expressed only in the mother cell compartment (Turner *et al.*, 1986) should help to clarify this problem.

The fall in β -galactosidase activity after about t_4 was presumed to be due to the onset of toluene-resistance in the developing spore, since the effect was reversed by germination, which leads to almost immediate loss of resistance properties (Setlow, 1981). Some of the *spo* mutant strains that do not develop toluene resistance appeared to over-express β -galactosidase in

comparison with *Spo*⁺ strains (particularly *spoIVC*, *spoIVD* and *spoVE* mutants; Table 2). Several other mutants that did not over-express the enzyme, as measured at *t*₄, nevertheless showed continued accumulation of activity, in contrast to the subsequent sharp fall in activity seen in the wild-type and in strains such as the *spoVA* mutants that develop some resistance to toluene (results not shown). It therefore seems likely that toluene-resistance affects the detection of β -galactosidase in the spore. However, toluene treatment does not appear to be necessary for the detection of β -galactosidase by MUG when expressed from some *spo* genes. We have compared the measured activities in time-course experiments with and without toluene treatment, for expression from both the *spoIIAA::lacZ* and *spoVAA::lacZ* fusion genes in wild-type host cells (results not shown). The time course for *spoIIA* expression was similar in both cases, whereas the detected activity for the *spoVA* expression was almost negligible in the absence of toluene treatment. Furthermore, similar results were obtained even for late blocked toluene-sensitive *spo* mutants. It therefore appears that access of MUG into the developing spore is affected by factors, such as changes in permeability, that are distinct from those involved in resistance to toluene. Alternatively, it is possible that the biochemical state of the intact (i.e. non-toluene-treated) developing spore prevents enzymic cleavage of the substrate. In any case, the effect of toluene treatment on measured enzyme activity may provide a useful means of determining the compartment in which other *spo::lacZ* fusion genes are expressed.

spoVA expression begins at about *t*_{2.5}, which corresponds approximately to the time at which the first sporulating cells in the population complete engulfment, i.e. reach stage III (Dawes *et al.*, 1969). Since *spoVA* expression appears to be confined to the spore compartment, it would be interesting to look at the effect of *spoVA* mutations upon subsequent events in sporulation. Synthesis of the acid-soluble spore proteins and of glucose dehydrogenase are likely to be spore compartment events (reviewed by Setlow, 1981). Neither of these is prevented by *spoVA* mutations (Johnson *et al.*, 1985; Errington & Mandelstam, 1984), but both events occur at about the same time as *spoVA* is expressed, and therefore they might just precede expression of *spoVA*. Alternatively, all of these genes could be controlled by a common regulator. Synthesis of dipicolinic acid, a compound which is found in the spore (Murrell *et al.*, 1969), normally begins at about *t*₄ but is prevented by mutations in *spoVA* (Errington & Mandelstam, 1984). However, the distribution of this compound may be misleading since Andreoli *et al.* (1975) have suggested that dipicolinic acid synthetase, the enzyme responsible for dipicolinic acid synthesis, is located exclusively in the mother cell compartment. The synthesis and assembly of the spore coat proteins has been studied in detail (Jenkinson *et al.*, 1981) and may be a good indicator of mother-cell-specific gene expression in *B. subtilis*. Synthesis of certain coat proteins begins at about *t*₂, whereas others begin to be synthesized later, mainly around *t*₃ or *t*₄. The proteins are deposited on the outside of the developing spore in a multi-layered structure, not following the order in which they are synthesized. One major coat protein, of *M*_r 36000 ('36K' protein), characteristically begins to be synthesized very late, from about *t*₆, and it is a major component of the outer layer of the mature spore coat. A *spoVA* mutant strain produced spore coats indistinguishable from those of the wild-type (Fig. 4). Since even the synthesis of the '36K' polypeptide as late as *t*₆ is unaffected by mutations in the *spoVA* operon, it seems that expression or non-expression of this operon may have no effect on the sequential expression of sporulation loci in the mother cell. The results presented here raise the possibility that expression of *spoVA* may represent an early event in a spore specific line of gene expression that is at least partially independent of events in the mother cell.

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