Use of a *lacZ* Gene Fusion to Determine the Dependence Pattern of Sporulation Operon *spoIIA* in *spo* Mutants of *Bacillus subtilis*

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A *spoIIA::lacZ* gene fusion has been used to investigate the dependence pattern of expression of the *spoIIA* operon during sporulation in *Bacillus subtilis*. β -Galactosidase activity, encoded by the hybrid gene, begins to appear about 30 to 60 min after the induction of sporulation. *spoIIA* expression is dependent upon the products of all of the known *spo0* loci but on none of the 'later' loci tested. The β -galactosidase activity falls after 1.5 h in *Spo*⁺ cells and in late-blocked mutants, but continued accumulation of the enzyme occurs in certain stage II mutants. Kinetic experiments suggest that the fall in activity may be, in part, the result of regulation at the level of translation. Mutations in several loci, *spo0J*, *spoIIIF* and *spoVIC*, delay expression of the operon by 1–3 h. The significance of these results in terms of models for the control of gene expression during sporulation is discussed.

INTRODUCTION

About 50 of the 70 or so sporulation (*spo*) loci predicted as an upper limit on statistical grounds to exist (Hranueli *et al.*, 1974) have been identified by mutation and genetic mapping (Piggot & Coote, 1976; Piggot *et al.*, 1981). The pleiotropic phenotypes produced by mutations in *spo* loci suggested that, to a first approximation, their expression is regulated in a linear dependent sequence (Mandelstam, 1976). Thus, a mutation in any one *spo* gene prevents the occurrence of all later sporulation-specific events but has no effect on earlier events in the process. Later work on the phenotypes of certain *spo* double mutants, however, indicated the possible existence of branched or parallel pathways of control (Coote & Mandelstam, 1973; Piggot & Coote, 1976). Since the products of most of the sporulation genes have not yet been identified it has been difficult to investigate the control of their expression. Thus, little is known about the timing of expression of specific sporulation genes, or of the factors controlling their expression.

One method that has been successfully used to overcome similar problems in other organisms is the fusion of genes to sequences that encode a product that is easy to assay; usually the gene for β -galactosidase (*lacZ*) from *Escherichia coli* is used (Casadaban *et al.*, 1983). More recently, this approach has been applied with some success to the study of sporulation genes (Zuber & Losick, 1983; Stephens *et al.*, 1984; Ferrari *et al.*, 1985), and in the preceding paper a convenient general method for the construction of *lacZ* gene fusions in *B. subtilis* is described (Errington, 1986). By this method, single copy chromosomal *lacZ* gene fusions can be constructed and subsequently be transferred to derivatives of the temperate *B. subtilis* bacteriophage ϕ 105. These phage derivatives can be readily transferred into various genetic backgrounds using a selectable chloramphenicol-resistance determinant.

The sporulation locus *spoIIA* has been extensively studied by classical genetic techniques (Yudkin & Turley, 1980, 1981; Errington & Mandelstam, 1983) and also at the molecular level (Lui *et al.*, 1982; Piggot *et al.*, 1984; Savva & Mandelstam, 1984; Fort & Piggot, 1984; Yudkin *et al.*, 1985). The locus, which is adjacent to a second sporulation locus, *spoVA* (Errington & Mandelstam, 1984), and near *lys-1* on the *B. subtilis* chromosome, contains three genes,

designated *A*, *B* and *C*, which are regulated as a polycistronic operon (Piggot *et al.*, 1984; Savva & Mandelstam, 1986). Gene *C* shows strong homology to the gene for the sigma subunit of RNA polymerase (Errington *et al.*, 1985).

In this paper a *lacZ* fusion to the *A* gene of *spoIIA*, constructed as described by Errington (1986), was used to investigate the expression of *spoIIA* during sporulation in the wild-type and in a variety of Spo⁻ mutants. Similar analyses of expression of the adjacent *spoVA* operon and of operons *spoIID* and *spoIIC* are described in the following papers (Errington & Mandelstam, 1986; Clarke *et al.*, 1986; Turner *et al.*, 1986). The results of a systematic study of the effects of mutations at other *spo* loci on the expression of *spoIIA* suggest that *spoIIA* is one of the first of the known sporulation-specific operons to be expressed.

Piggot *et al.* (1985) have described preliminary results of a *lacZ* fusion analysis of *spoIIA* expression using a rather different system.

METHODS

Bacterial strains. These are listed in Table 1.

Media. Hydrolysed casein growth medium and sporulation medium were prepared as described by Sterlini & Mandelstam (1969). Solid media were Oxoid nutrient agar and lactate/glutamate minimal agar (Piggot, 1973). L-Tryptophan was added to minimal media to a final concentration of 20 µg ml⁻¹.

Growth and sporulation. Cultures, at a density of about 0.25 mg dry wt ml⁻¹, were induced to sporulate by the resuspension method of Sterlini & Mandelstam (1969). Times (h) after resuspension in sporulation medium are denoted *t*₀, *t*₁, etc. All liquid cultures were grown at 37 °C with shaking.

Transformation. DNA extracted from donor cultures as described by Ward & Zahler (1973) was used (final concentration 0.1 µg ml⁻¹) to transform the recipient strain, CU267, made competent by the method of Anagnostopoulos & Spizizen (1961). Ilv⁺ Leu⁺ transformants were selected on lactate-glutamate minimal agar containing tryptophan, and Spo⁻ congressants were isolated.

Transduction. Crude lysates of bacteriophage φ105 and its derivative φ105J19, prepared as described by Jenkinson & Mandelstam (1983), were mixed in equal proportions, and a 20 µl portion, containing about 10⁹ p.f.u., was spotted onto a lawn of the recipient strain on Oxoid nutrient agar. After 24 h at 37 °C, lysogenic bacteria growing in the infected area of the plate were streaked for single colonies on nutrient agar containing chloramphenicol (5 µg ml⁻¹).

spoIIAA::lacZ gene fusion. In the preceding paper the construction of a chromosomal *spoIIAA::lacZ* gene fusion and a transducing phage, φ105J19, is described (Errington, 1986). The latter can be used to transfer the *spoIIA* regulatory sequences and the gene fusion to other bacterial strains by selecting for transduction to chloramphenicol resistance. The structure of the DNA insert in this phage is shown in Fig. 1. The hybrid gene consists of the first 23 codons of the *spoIIAA* gene, followed by a five codon spacer region of 'synthetic' origin, and then the whole of the *lacZ* gene except for the first eight codons (Kalnins *et al.*, 1983).

β-Galactosidase assays. Samples (0.5 ml) from sporulating cultures were harvested (Beckman Microfuge; 12–15 s) and the cells were washed in 1 ml ice-cold Tris/HCl (50 mM, pH 7.5). The cell pellet was rapidly frozen in liquid N₂ and stored at -70 °C. The cells were thawed by resuspension in 600 µl Z buffer (50 mM-sodium phosphate, pH 7.0; 50 mM-2-mercaptoethanol; 10 mM-KCl; 1 mM-MgSO₄) (Miller, 1972) and then permeabilized using the cold Tris/toluene method of Fisher *et al.* (1975).

For assays using *o*-nitrophenyl-β-D-galactopyranoside (Sigma) ('ONPG assays'), the method of Miller (1972) was used. One (ONPG) unit of β-galactosidase produces 1 nmol *o*-nitrophenol min⁻¹ at 28 °C. For assays using 4-methylumbelliferyl-β-D-galactopyranoside (Sigma) ('MUG assays'), substrate (200 µl of a solution containing 40 µg ml⁻¹ in Z buffer) was added, and the reaction was stopped after 15 min at 30 °C by the addition of 400 µl 1 M-Na₂CO₃. The suspension was cleared (Beckman Microfuge; 5 min) and the concentration of the product, 4-methylumbelliferone, in 1 ml supernatant was determined by fluorescence spectrophotometry (frequency of excitation, 365 nm; frequency of detection, 450 nm) using a range of standard solutions (0.01, 0.1, 1.0 and 10 µg ml⁻¹). One (MUG) unit of β-galactosidase catalyses the production of 1 nmol 4-methylumbelliferone min⁻¹. (One MUG unit represents about 10 ONPG units.)

RESULTS

β-Galactosidase production by strains carrying a spoIIA::lacZ fusion

The results of preliminary work on strains producing β-galactosidase from a *spoIIA::lacZ* gene fusion were complicated by a β-galactosidase-like activity in the wild-type strain MB75 (and other strains), which contains no *lacZ* gene. Development of β-galactosidase activity in sporulating cultures of strains MB75 and 613 was determined using the ONPG assay (Fig. 2a).

Table 1. *Bacillus subtilis* strains

Strain	Genotype*	Origin			
Strains containing a <i>spoIIA::lacZ</i> fusion					
613 (Spo ⁻)	<i>lys-1 metC3 tal-1 spoIIAA::lacZ-cat</i>	Errington (1986)			
SG36 (Spo ⁺)	<i>lys-1 metC3 tal-1 (φ105J19 spoIIAA::lacZ-cat)</i>				
612.3 (Spo ⁻)	<i>trpC2 ΔspoIIA4 (φ105J19 spoIIAA::lacZ-cat)</i>	Transduction of strain 612.2 with bacteriophage φ105J19 (Errington, 1986) by selection for Cm ^R			
613.1 (Spo ⁻)	<i>trpC2 spoIIAA::lacZ-cat</i>	Transformation of strain SG38 with DNA from strain 613 and selection for Cm ^R			
613.2 (Spo ⁺)	<i>metC3 lys-1 spoIIAA::lacZ-cat (φ105DS1 spoIIA⁺ spoVA⁺)</i>	Transduction of strain 613.1 with bacteriophage φ105DS1 (Savva & Mandelstam, 1984) by selection for Spo ⁺			
Spo ⁺ strains					
MB75	<i>lys-1 metC3 tal-1</i>	Laboratory stock S. A. Zahler, Cornell University, USA Transformation of strain CU267 to <i>Ilv⁺ Leu⁺</i> with DNA from strain 168			
168	<i>trpC2</i>				
CU267	<i>trpC2 ilvB2 leuB16</i>				
SG38	<i>trpC2</i>				
Strain	<i>spo</i> genotype	Origin of <i>spo</i> mutation†	Strain	<i>spo</i> genotype	Origin of <i>spo</i> mutation†
Spo ⁻ strains (all are <i>trpC2</i>)			Spo ⁻ strains (all are <i>trpC2</i>)		
1.5	<i>spoIIAC1</i>	E1; Piggot (1973)	165.3	<i>spoIVB165</i>	P7; Coote (1972)
2.12	<i>spoIIIB2</i>	A3; Piggot (1973)	221.1	<i>spo0F221</i>	<i>spoF221</i> ; Hoch & Mathews (1973)
17‡	<i>spo0H17</i>	E3; Waites <i>et al.</i> (1970)	224.1	<i>spoVF224</i>	DG47; Coote (1972)
23.4	<i>spoIVC23</i>	E31; Waites <i>et al.</i> (1970)	298.4	<i>spoIID298</i>	P9; Coote (1972)
25.7	<i>spoIVG25</i>	E33; Waites <i>et al.</i> (1970)	484.2	<i>spo0E11</i>	<i>spoE11</i> ; Hoch & Mathews (1973)
36‡	<i>spoIIIE36</i>	NG1.67; Piggot (1973)	485‡	<i>spo0G14</i>	14UL; Ionesco <i>et al.</i> (1970)
43.6	<i>spo0A43</i>	NG6.21; Piggot (1973)	486‡	<i>spo0D8</i>	8H; Ionesco <i>et al.</i> (1970)
48.7	<i>spoIIE48</i>	NG9.3; Piggot (1973)	488.8	<i>spo0B136</i>	<i>spoB136</i> ; Hoch & Mathews (1973)
55.3	<i>spoIIG55</i>	NG12.12; Piggot (1973)	496.1	<i>spoIIIC94</i>	94U; Ionesco <i>et al.</i> (1970)
65‡	<i>spoIIIA65</i>	NG17.17; Piggot (1973)	497.1	<i>spoIIID83</i>	83U; Ionesco <i>et al.</i> (1970)
67‡	<i>spoIVA67</i>	NG17.23; Piggot (1973)	498‡	<i>spoIVE11</i>	11T; Ionesco <i>et al.</i> (1970)
85.3	<i>spoVE85</i>	Hranueli <i>et al.</i> (1974)	513.3	<i>spoVIA513</i>	Jenkinson (1981)
88.7	<i>spoIVF88</i>		516‡	<i>spoVH516</i>	Hill (1983)
89.10	<i>spoVAE89</i>		517‡	<i>spoVJ517</i>	
91‡	<i>spoVB91</i>		520.3	<i>spoVIB520</i>	Jenkinson (1983)
92.5	<i>spoIVD92</i>		522.2	<i>gerE36</i>	Moir (1981)
93.2	<i>spo0J93</i>		562.5	<i>spoIIAA562</i>	Errington & Mandelstam (1983)
131.5	<i>spoIIB131</i>	Z3; Coote (1972)	590.4	<i>spoIIIF590</i>	Lamont & Mandelstam (1984)
134‡	<i>spoVC134</i>	Z10A; Coote (1972)	610.2	<i>spoVIC610</i>	James & Mandelstam (1985)
141‡	<i>spo0K141</i>	Z31; Coote (1972)	612.2	<i>ΔspoIIA4</i>	4Z; Ionesco & Schaeffer (1968)
156‡	<i>spoVD156</i>	W10; Coote (1972)			

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

† Original mutant strain designation and reference.

‡ These strains have a *trpC2* genotype but have various, and in some cases obscure, origins. The remaining strains are isogenic: all were derived from strain CU267 by congression of the *spo* mutation following selection for Ilv⁺ Leu⁺.

Although there is a clear difference between the activities in both strains after t_1 , the wild-type strain MB75 shows a low degree of activity that appears to be sporulation-related, i.e. it begins to be formed after t_0 . Fortunately, an alternative fluorimetric assay for β-galactosidase was available, based on the hydrolysis of MUG (see Methods). This substrate was more specific for the *lacZ*-encoded β-galactosidase activity and it was used in all subsequent experiments.

Fig. 1. Structure of phage $\phi 105J19$ carrying the *spoIIAA::lacZ* gene fusion. The lower part of the figure shows the region of the *B. subtilis* chromosome containing the *spoIIA* operon (three genes) and the *spoVA* operon (five genes). Phage $\phi 105J19$ contains a fragment of chromosomal DNA covering these operons but the central portion of the insert has been replaced by the *E. coli lacZ* gene (hatched box) and a chloramphenicol-resistance gene (filled box). The insertion is arranged so that the region encoding the N-terminus of the *lacZ* gene is fused, in frame, to the beginning of the *spoIIAA* gene (Errington, 1986). The remainder of the *spoIIA* operon and most of the *spoVA* operon are deleted in the phage. Part of the DNA sequence around the junction of the *spoIIAA* and *lacZ* genes is shown at the top of the figure, along with the amino acid translation in standard one-letter code. The putative ribosome binding site for the *spoIIAA* gene is underlined (Fort & Piggot, 1984).

Fig. 2. β -Galactosidase production during sporulation by strains SG36 (●), 613 (○) and MB75 (□), as measured using an assay based on hydrolysis of (a) ONPG or (b) MUG.

Fig. 3

Fig. 4

Fig. 3. Rate of degradation of β -galactosidase encoded by the *spoIIA::lacZ* fusion gene in *Spo*⁺ sporulating cells. A 50 ml culture of strain SG36 was induced to sporulate and samples were taken at intervals to assay β -galactosidase activity (MUG assay). After 60, 75, 90 and 120 min, 5 ml portions of culture were treated with chloramphenicol (100 μ g ml⁻¹) to prevent further protein synthesis. The subsequent rate of degradation of β -galactosidase in these samples was determined by measuring the remaining activity at 3 min intervals. $\circ$, Control culture; $\bullet$, chloramphenicol-treated culture.

Fig. 4. β -Galactosidase production during sporulation in strains carrying a *spoIIA::lacZ* gene fusion on the ϕ 105J19 prophage. Strains lysogenic for ϕ 105J19 and carrying no *spo* mutation ($\circ$) or mutations in the *spo0J* ($\bullet$), *spoIIIF* ($\blacksquare$) or *spoVIC* ($\triangle$) loci were induced to sporulate as described in Methods. At intervals, samples were taken and assayed for β -galactosidase activity (MUG assay).

The course of development of β -galactosidase activity in sporulating cultures carrying a *spoIIA::lacZ* fusion in either the chromosomal or the prophage location is compared in Fig. 2(b). Note that the background activity produced by strain MB75 (wild-type control) is negligible in comparison with the activities produced by the other two strains. In both of these, β -galactosidase is induced between $t_{0.5}$ and t_1 and reaches a peak at about $t_{1.5}$. Although there was some day-to-day variation in the height of the peak, the time of induction was very reproducible. Strain SG36, which is phenotypically *Spo*⁺ and carries the *lacZ* gene fusion on the ϕ 105J19 prophage, showed a subsequent fall in activity, reaching the background activity of the wild-type at about t_4 . By contrast, strain 613, which is phenotypically *SpoIIA*⁻ because of the insertion of *lacZ* into the *spoIIA* locus on the chromosome, reached a plateau at about $t_{1.5}$, began to increase again at about t_3 , and was still increasing slowly at t_6 . The reasons for these differences were not clear, but the effect was shown to depend upon the *SpoIIA* phenotype of the strain rather than upon the location of the *spoIIA::lacZ* fusion (chromosomal or prophage). Strain 613.2, in which the lesion produced by the chromosomal *spoIIA::lacZ* fusion is complemented by the ϕ 105DS1 prophage (Savva & Mandelstam, 1984), showed a similar fall in β -galactosidase activity after $t_{1.5}$. Strain 612.3, which carries the *spoIIA::lacZ* fusion on the ϕ -105J19 prophage but is phenotypically *SpoIIA*⁻ as a result of an extensive deletion in the *spoIIA-spoVA* region (mutation *spoIIA4*; Piggot *et al.*, 1984), showed an increase in activity after $t_{1.5}$ which was similar to that of strain 613 (data not shown).

Effect of chloramphenicol on β -galactosidase time courses

The fall in β -galactosidase activity that occurred after $t_{1.5}$ in *Spo*⁺ strains might have been due to an increase in the rate of degradation of the enzyme, or to a decrease in its rate of synthesis, or to both. To distinguish between these possibilities, cells of strain SG36 were induced to sporulate, and at t_1 , $t_{1.25}$, $t_{1.5}$ and t_2 , portions were treated with chloramphenicol (100 μ g ml⁻¹; the single copy *cat* gene provides resistance to 5 μ g chloramphenicol ml⁻¹ only), to inhibit

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

Phage ϕ 105J19, carrying a *spoIIAA : : lacZ* fusion, was transduced into a series of otherwise isogenic strains, carrying *spo* mutations. Each strain in turn was induced to sporulate and samples were taken for the MUG assay of β -galactosidase. Results shown are mean activities in samples at $t_{1.5}$ and t_4 . The wild-type *spo*⁺ strain (SG38) containing phage ϕ 105J19 produced 0.58 units β -galactosidase ml⁻¹ at $t_{1.5}$ and 0.17 units ml⁻¹ at t_4 (means of five determinations). The activities produced by strain SG38 with no *lacZ* fusion were 0.013 units ml⁻¹ at $t_{1.5}$ and 0.032 units ml⁻¹ at t_4 .

Mutation	β -Galactosidase activity (units ml ⁻¹)		No. of determinations	Mutation	β -Galactosidase activity (units ml ⁻¹)		No. of determinations
	$t_{1.5}$	t_4			$t_{1.5}$	t_4	
<i>spo0A43</i>	0.016	0.037	2	<i>spoIV A67</i>	0.29	0.080	3
<i>spo0B136</i>	0.023	0.054	2	<i>spoIV B165</i>	0.12	0.19	3*
<i>spo0D8</i>	0.024	0.034	2	<i>spoIV C23</i>	0.29	0.15	3
<i>spo0E11</i>	0.018	0.015	2	<i>spoIV D92</i>	0.43	0.060	2
<i>spo0F221</i>	0.021	0.050	2	<i>spoIV E11</i>	1.2	0.074	2
<i>spo0G14</i>	0.024	0.054	2	<i>spoIV F88</i>	0.23	0.14	3
<i>spo0H17</i>	<0.01	0.018	2	<i>spoIV G25</i>	0.57	0.14	3
<i>spo0J93</i>	0.048	0.25	2	<i>spoV A89</i>	0.44	0.34	2
<i>spo0K141</i>	0.013	0.050	2	<i>spoV B91</i>	0.49	0.21	2
<i>spoIIA A562</i>	0.41	0.38	2	<i>spoV C134</i>	0.61	0.46	2
<i>spoIIA C1</i>	0.44	0.51	2	<i>spoV D156</i>	0.60	0.23	3
<i>spoIIB131</i>	0.36	0.17	3	<i>spoV E85</i>	1.1	0.19	2
<i>spoIID298</i>	0.43	0.11	2	<i>spoV F224</i>	0.26	0.14	2
<i>spoIIE48</i>	0.67	0.77	3	<i>spoV H516</i>	0.80	0.26	3
<i>spoIIG55</i>	0.69	0.58	3	<i>spoV J517</i>	0.50	0.16	3
<i>spoIIIA65</i>	0.18	0.22	4	<i>spoVIA513</i>	1.0	0.19	3
<i>spoIIB2</i>	0.65	0.13	2	<i>spoVIB520</i>	0.38	0.22	2
<i>spoIIIC94</i>	0.24	0.23	4	<i>spoVIC610</i>	0.085	0.20	3
<i>spoIIID83</i>	1.8	0.52	2	<i>gerE36</i>	0.85	0.14	2
<i>spoIIIE36</i>	1.2	0.12	2				
<i>spoIIIF590</i>	0.032	0.23	4				

* The $t_{1.5}$ sample was only assayed twice.

protein synthesis (Jenkinson *et al.*, 1980). Samples from each portion were assayed for β -galactosidase at 3 min intervals to measure the rate of degradation of the enzyme. The results (Fig. 3) show that the rapid rate of degradation remains relatively constant from t_1 to t_2 . Thus, the fall in activity that occurs after about $t_{1.25}$ in the control culture must signify a reduction in the rate of synthesis of β -galactosidase. If this interpretation is correct it has interesting implications for the regulation of spore formation, because Northern blot experiments (Savva & Mandelstam, 1985, 1986) show that the mRNA of the *spoIIA* operon continues to be present until at least t_4 (see Discussion).

Effect of various spo mutations on expression of spoIIA

To investigate the control of *spoIIA* expression by other *spo* loci a set of isogenic strains was constructed carrying mutations in each of the known *spo* loci. Phage ϕ 105J19, carrying the *spoIIA : : lacZ* fusion and all of the sequences necessary for its expression, was transferred into each of the *spo* mutant strains by selecting transductants that were resistant to chloramphenicol. Each of the derivative strains was subsequently induced to sporulate and samples were taken, for β -galactosidase assays, at t_0 , $t_{1.5}$ and t_4 . The last of these samples was taken to determine whether the characteristic fall in activity after $t_{1.5}$ seen in Spo⁺ strains, but not in SpoIIA⁻ strains, occurred. The results (Table 2) are mean activities from at least two separate sporulation experiments using the MUG assay. Preliminary data obtained with the ONPG assay were in accordance with those shown in Table 2.

Mutations at most sporulation loci gave unambiguous results. In general, β -galactosidase induction had occurred by $t_{1.5}$ in strains mutated in any of the stage II or 'later' loci, but not in any of the stage 0 loci. A clear distinction was seen between the stage 0 mutants and the 'later' mutants. The assessment of activities at t_4 was more difficult. However, most of the strains that displayed moderate activities at $t_{1.5}$ (> 0.2 units ml^{-1}) showed a substantial decrease by t_4 . The main exceptions were strains carrying mutations in the *spoIIA* (*A* and *C* genes), *spoIIE* and *spoIIG* loci. The differences between these stage II mutants and the other mutants was confirmed by more detailed time-course experiments that were done with strains mutated in most of the sporulation loci (data not shown). Mutations in several loci, *spo0J*, *spoIIIF* and *spoVIC*, had a more complex effect on expression. Strains carrying these mutations had small or negligible activities at $t_{1.5}$ but substantial activities at t_4 . Time-course experiments showed that in each case the initial appearance of β -galactosidase was delayed by between 1 and 3 h (Fig. 4). Moreover, the extent of the delay was reproducible and characteristic for each particular mutation. None of the other sporulation mutants displayed this sort of anomalous behaviour, though rather high peak activities were observed in *spoIIID*, *spoIIIE*, *spoIVE* and *spoVE* mutants.

DISCUSSION

Initial characterization of *spoIIA::lacZ* expression by an assay based on hydrolysis of ONPG was complicated by the presence of an indigenous β -galactosidase-like activity, which was induced at the onset of sporulation. This almost certainly does not represent a sporulation specific event since its induction occurs even in a *spo0A* mutant (results not shown). The β -galactosidase activity due to the *lacZ* gene was clearly resolved from the background activity by an assay based on the fluorogenic substrate MUG. An accompanying paper (Clarke *et al.*, 1986) gives altered MUG assay conditions that reduce the overall sensitivity but further enhance the difference between 'real' and 'background' activities.

β -Galactosidase production from the hybrid *spoIIA::lacZ* gene begins within the first hour of sporulation and, in the wild-type, reaches a peak after about 1.5 h (Fig. 2). The subsequent fall in activity suggested that the enzyme was being degraded, presumably by proteolysis, since several proteases are known to be present in sporulating cells (e.g. Sastry *et al.*, 1983; Jenkinson & Lord, 1983). However, the experimental results (Fig. 3) clearly show that the fall in activity after $t_{1.5}$ is not due to an increased rate of proteolysis and that, therefore, part of the fall is probably the result of a decrease in the rate of enzyme synthesis. Moreover, Savva & Mandelstam (1985, 1986) have demonstrated that mRNA for the *spoIIA* operon can be detected at least until t_6 in wild-type sporulating cells. Taken together, these results indicate that the reduction in synthesis of the *spoIIA::lacZ* product after $t_{1.5}$ may be due to regulation at the level of translation. Well-documented mechanisms for translational control are rare in prokaryotes. They usually involve genes whose products are either involved in the machinery of protein synthesis, for example ribosomal proteins (Nomura *et al.*, 1984) and amino acyl-tRNA-synthetases (Springer *et al.*, 1985), or genes whose products confer resistance to antibiotics, such as erythromycin (Horinouchi & Weisblum, 1980) or chloramphenicol (Duvall *et al.*, 1985), that block protein synthesis.

Clearly, the finding that there may be translational control further complicates the general problem of the regulation of spore formation; however, more work is needed to demonstrate directly that there really is control of this type and to elucidate its mechanism.

Whatever the reason for the fall in activity of β -galactosidase, the *spoIIA*, *spoIIE* and *spoIIG* loci are required for it to occur since mutations in these loci cause continued accumulation of β -galactosidase after $t_{1.5}$. It is interesting to note that the *spoIIAC* and *spoIIG* genes both encode proteins similar to the sigma subunit (σ) of RNA polymerase (Errington *et al.*, 1985; Stragier *et al.*, 1984, 1985). Since a *spoIIA* mutation prevents synthesis of σ^{29} (Trempy *et al.*, 1985a), which is now known to be a product of the *spoIIG* locus (Trempy *et al.*, 1985b), we can infer that *spoIIA* and *spoIIG* are expressed sequentially. Furthermore, it has also been suggested (Trempy *et al.*, 1985a) that a product of the *spoIIE* locus is necessary for processing an inactive precursor

protein, P₃₁, into the mature form of σ^{29} . These results combined with those obtained in experiments with cells containing *spoIID::lacZ* fusions suggest a tentative linear order of expression for the genetic loci controlling stage II (see Clarke *et al.*, 1986).

Of the loci tested, only mutations in the *spo0* loci (with the possible exception of *spo0J*) completely abolished expression of *spoIIAA::lacZ*. Since the *spo0* loci seem to be expressed during vegetative growth and may be concerned with sensing the nutritional state of the cell (Losick & Youngman, 1984), the *spoIIA* operon seems to be among the first of the sporulation-specific loci to be expressed.

Several loci, *spo0J*, *spoIIIF* and *spoVIC*, had a more complex effect on *spoIIAA::lacZ* expression. They caused a reproducible delay in expression of between 1 and 3 h (Fig. 4). Although the amount of β -galactosidase produced by the *spo0J* strain was small compared with that produced by the *spoIIIF* and *spoVIC* strains, it was, nevertheless, considerably enhanced compared with that produced by the other *spo0* strains (about fivefold; Table 2). The strain carrying the *spoVIC* mutation was isolated recently and it is characterized by defects in both germination and lysozyme resistance (James & Mandelstam, 1985). There was also a delay in the occurrence of sporulation marker events, beginning with alkaline phosphatase at about t_2 . The delay caused by mutations in the *spoVIC* and *spoIIIF* loci was unexpected, and the implication is that the products of these loci intervene in the process of sporulation before the induction of *spoIIA* expression. These findings further emphasize that the time at which a *spo* gene is expressed may be much earlier than the time at which the phenotype of a mutation in that gene is manifested (Wood, 1972). Two examples of this type of phenomenon have been described previously: (i) some proteins of the outer spore coat are synthesized several hours before they are laid down (Jenkinson *et al.*, 1981); (ii) the *spoVG* gene, in which mutations give rise to a defect in cortex formation (Rosenbluh *et al.*, 1981), is expressed at the onset of sporulation under some conditions (Segall & Losick, 1977). Similar delays in expression of *spoVAA::lacZ* and *spoIID::lacZ* fusions by mutations in *spoVIC* and *spoIIIF* but not *spo0J* are reported in the following papers (Errington & Mandelstam, 1986; Clarke *et al.*, 1986).

The presentations here and in the two following papers (Errington & Mandelstam, 1986; Clarke *et al.*, 1986) represent the initial results of a survey of the regulation of a series of *spo* loci undertaken by making gene fusions to *lacZ*. Another accompanying paper (Turner *et al.*, 1986) shows that while the *spo* loci governing stage II probably are regulated as a linear dependent sequence (see Mandelstam, 1969, 1976), there is a branch in the pathway at a later stage. A further complication is introduced by the fact that at least one sporulation locus, *spoVA*, is expressed only in the spore compartment (Errington & Mandelstam, 1986).

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