Variety of Sporulation Phenotypes Resulting from Mutations in a Single Regulatory Locus, *spoIIA*, in *Bacillus subtilis*

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Closely linked mutations in either of the two putative genes of the sporulation locus *spoIIA* can affect, in quite diverse ways, spore incidence, the production of alkaline phosphatase and DNAase, and the stability of the cells in sporulation medium. It is concluded that the locus has a regulatory function affecting the activation or induction of at least two, and possibly more, sporulation-associated operons.

INTRODUCTION

Alkaline phosphatase and DNAase are two extracellular enzymes whose production is associated with stage II of sporulation in *Bacillus subtilis*. Mutants blocked early in stage II produce neither of the enzymes; those blocked later produce both (Waites *et al.*, 1970; Piggot & Coote, 1976; Akrigg & Mandelstam, 1978; Young & Mandelstam, 1979). It is not known whether they play a direct role in sporulation or whether they belong to a category of secondary events (Coote & Mandelstam, 1973). The question could be settled by isolating mutants in which the structural genes for the enzymes are damaged, but, in spite of considerable effort, no such mutants have been found (Grant, 1974; Porter & Mandelstam, 1982).

The specific associations of alkaline phosphatase and DNAase with stage II could be explained in one of two ways. First, the genes for the enzymes might be included in operons that lie in the main sequence whose successive expression is necessary for the formation of the spore. Alternatively, they might lie on operons unconnected with the main sequence. These might be switched on either by the same regulator molecule that activates an operon in the main sequence or by some other molecule such as an intermediary metabolite generated by some biochemical change, e.g. enzymic action of a protein coded for in the main sequence.

This paper describes the phenotypic consequences of a number of closely linked mutations in the *spoIIA* locus. Earlier work had shown that mutations in this locus result not only in asporogeny or oligosporogeny but also in failure to produce alkaline phosphatase (Coote, 1972*a*; Piggot, 1973). It was subsequently shown that there is also a failure to produce extracellular DNAase (Akrigg & Mandelstam, 1978). None of the later sporulation events, e.g. production of glucose dehydrogenase or dipicolinic acid, occurred in these mutants. These results all suggest that *spoIIA* is a 'main-stream' locus. More recently, Yudkin & Turley (1981) showed that mutations in one part of the locus could be partly suppressed by another mutation elsewhere in the same locus and that this altered not only the degree of oligosporogeny but the rate at which sporulation occurred. Furthermore, Liu *et al.* (1982) cloned part of the *spoIIA* locus on a plasmid and demonstrated that this plasmid would complement mutations that had been shown by Yudkin & Turley (1981) to lie at one end of the locus, whereas mutations lying at the other end could not be complemented. It thus appears that the *spoIIA* locus contains at least two separate genes.

The experiments described in this paper show that closely linked mutations within either of the putative genes can produce quite different effects on alkaline phosphatase, DNAase and sporulation. They also affect in different ways the stability of the cells in sporulation medium.

Table 1. *Bacillus subtilis* strains

Strain	Genotype	Previous designation and reference
<i>spo</i> ⁺ strains		
168	<i>trpC2</i>	
MB68	<i>lys-1 phe-12 rif-2 tal-1</i>	
MB75	<i>lys-1 metC3 tal-1</i>	
MY2016	<i>hisH2 lys-1 rif sul</i> ^R	Yudkin & Turley (1980)
<i>spo</i> mutants		
1	<i>trpC2 spoIIA1</i>	E1 (Piggot, 1973)
37	<i>trpC2 spoIIA37</i>	NG1.82 (Piggot, 1973)
42	<i>trpC2 spoIIA42</i>	NG6.13 (Piggot, 1973)
50	<i>trpC2 spoIIA50</i>	NG11.2 (Piggot, 1973)
63	<i>trpC2 spoIIA63</i>	NG16.2 (Piggot, 1973)
69	<i>trpC2 spoIIA69</i>	NG18.6 (Piggot, 1973)
Constructed strains		
Strain	Relevant genotype	Construction
SG16	<i>hisH2 spo</i> ⁺	MY2016 transformed to Lys ⁺ using DNA from strain 168
1.1	<i>hisH2 spoIIA1</i>	MY2016 transformed to Lys ⁺ using the appropriate <i>spo</i> donor above, and <i>spo</i> transformants isolated
37.1	<i>hisH2 spoIIA37</i>	
42.1	<i>hisH2 spoIIA42</i>	
50.2	<i>hisH2 spoIIA50</i>	
63.6	<i>hisH2 spoIIA63</i>	
69.4	<i>hisH2 spoIIA69</i>	Mutants isolated by transformation of MY2016 to Lys ⁺ using mutagenized DNA from strain 168 (see Results)
560	<i>hisH2 spo560</i>	
561	<i>hisH2 spo561</i>	
562	<i>hisH2 spo562</i>	
563	<i>hisH2 spo563</i>	
564	<i>hisH2 spo564</i>	MB75 transformed to Met ⁺ using the appropriate <i>hisH2 spo</i> donor above, and Spo ⁻ congressants isolated
565	<i>hisH2 spo565</i>	
1.3	<i>lys-1 spoIIA1</i>	
37.2	<i>lys-1 spoIIA37</i>	
42.2	<i>lys-1 spoIIA42</i>	
50.3	<i>lys-1 spoIIA50</i>	
63.6	<i>lys-1 spoIIA63</i>	
69.6	<i>lys-1 spoIIA69</i>	
560.1	<i>lys-1 spo560</i>	
561.1	<i>lys-1 spo561</i>	
562.1	<i>lys-1 spo562</i>	
563.1	<i>lys-1 spo563</i>	
564.1	<i>lys-1 spo564</i>	
565.1	<i>lys-1 spo565</i>	

The results can be most simply explained by assuming that the locus codes for a regulator protein that plays an essential part in the main sporulation pathway and that the same regulator also controls the production of the two extracellular enzymes and a factor (a lytic enzyme?) affecting the stability of the cells.

METHODS

Organisms. The bacterial strains used are shown in Table 1.

Media. Hydrolysed casein growth medium and sporulation medium were as described by Sterlini & Mandelstam (1969). Penassay broth was from Oxoid. Solid media were Oxoid nutrient agar and lactate/glutamate minimal agar (Piggot, 1973). Where appropriate the latter was supplemented with L-amino acids at a final concentration of 200 µg ml⁻¹.

Growth and sporulation. Cultures were grown at 37 °C with shaking except where otherwise stated. Culture density was measured turbidimetrically at 600 nm with a Unicam SP600 spectrophotometer and readings were converted to mg dry wt bacteria ml⁻¹. Sporulation was achieved using the resuspension technique of Sterlini & Mandelstam (1969). Times (h) after resuspension in sporulation medium are denoted *t*₁, *t*₂, *t*₃, etc.

Transformation. DNA was extracted from donor cultures as described by Ward & Zahler (1973), except where otherwise indicated, and was used to transform recipient cultures made competent by the method of Anagnostopoulos & Spizizen (1961). Prototrophic recombinants were selected on lactate/glutamate minimal agar

supplemented with the appropriate amino acids. Spo⁺ transformants were selected on Schaeffer's agar (Schaeffer *et al.*, 1965) by treatment with chloroform vapour as described by Hoch (1971).

Directed mutagenesis and mutant isolation. Mutations in the *spoIIA* locus were obtained by making use of its linkage (about 30%) to *lys-1* by transformation (Yudkin & Turley, 1981). Heavily mutagenized DNA was prepared from strain 168 (Spo⁺) as follows. A culture in penassay broth (200 ml) was grown to a density of about 0.15 mg dry wt ml⁻¹. The cells were harvested by centrifugation and resuspended in 10 ml sporulation medium containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 µg ml⁻¹). After 40 min incubation with shaking the cells were harvested by centrifugation, washed twice in sporulation medium, and resuspended in 200 ml penassay broth. After further incubation (1 h, with shaking) DNA was extracted using the method of Marmur (1961). The final yield of DNA was estimated to be about 36 µg by measurement of absorption at 260 nm on a Unicam SP800 spectrophotometer.

This DNA was used at a final concentration of 0.1 µg ml⁻¹ to transform MY2016 to Lys⁺ as described above. After incubation for 3 to 4 d it was possible to identify sporulation mutants on the basis of their failure to produce the characteristic brown pigment associated with sporulation. Potential mutants were purified by repeatedly streaking them on Lys⁺ selective medium. Microscopic examination of nutrient agar cultures after 3 d allowed those mutants that were highly oligosporogenous to be identified and discarded. In the remaining strains the *spo* mutations were tested for linkage to *lys-1* by transformation of strain MB68 to Lys⁺. Those that showed less than 10% linkage were also discarded.

Mapping. Three-factor transformation crosses were used for two purposes: firstly, to show that the new mutations lay on the same side of *lys-1* and in or near the *spoIIA* locus; secondly, to order different mutations in the *spoIIA* locus with respect to *lys-1*. The basis was as follows. Suppose that *spo-X* and *spo-Y* (below) represent two different mutations, one of which, *spo-X*, is known to lie in the *spoIIA* locus, while the other, *spo-Y*, shows similar transformation linkage to *lys-1* but may lie on either side of it. There are three possible positions for *spo-Y*:

These three possible orientations can be distinguished using reciprocal three-factor crosses as follows. Cross A: Recipient, *lys-1 spo-X*; donor, Lys⁺ *spo-Y*. Cross B: Recipient, *lys-1 spo-Y*; donor, Lys⁺ *spo-X*. In both crosses, Lys⁺ transformants can be selected and scored as Spo⁺ or Spo⁻ on the basis of colony pigmentation. If the order (1) above is correct then both crosses should give about 30% Spo⁺ transformants among the Lys⁺ transformants because *spoIIA* is 30% linked to *lys*. However, if either (2) or (3) represents the correct order, then the proportions of Spo⁺ transformants in both crosses would be greatly reduced. If both *spo-X* and *spo-Y* lie on the same side of *lys-1*, their relative positions can be inferred by comparing the ratios of Spo⁺/Lys⁺ among the transformants in the reciprocal crosses. In one cross, two crossovers would be required to give Spo⁺ transformants, whereas in the other, four crossovers would be necessary.

The map distance between two mutations can be estimated by dividing the frequency of Spo⁺ transformants by the frequency of Spo⁺ transformants using DNA from an isogenic Spo⁺ donor (strain SG16). The resulting value is the recombination index (RI) (Lacks & Hotchkiss, 1960) as described by Coote (1972*b*).

$$RI = \frac{\text{Frequency of Spo}^+ \text{ among Lys}^+ \text{ transformants (spo DNA)}}{\text{Frequency of Spo}^+ \text{ among Lys}^+ \text{ transformants (spo}^+ \text{ DNA)}}$$

Isolation of plasmid DNA. *Escherichia coli* strain SL3030 (Liu *et al.*, 1982), carrying the plasmid pHM2, was kindly provided by P. J. Piggot. Plasmid DNA was purified from this strain by the method of Guerry *et al.* (1973) after amplification using 300 µg spectinomycin ml⁻¹ (Klein *et al.*, 1980).

DNAase detection agar. This was prepared as described by Porter & Mandelstam (1982). DNAase detection plates were inoculated from overnight nutrient agar cultures. Production of DNAase was indicated by a halo of decolorization of the medium around the inoculum. Results were recorded after 2 to 3 d at 37 °C.

Enzyme assays. Alkaline phosphatase was assayed using a variation of the method of Glenn & Mandelstam (1971). Culture (1.5 ml) was mixed with 1 ml of *p*-nitrophenol phosphate (Sigma) (1 mg ml⁻¹ in 1 M-Tris/HCl pH 8.0) and incubated at 30 °C. When the colour (*A*₄₁₀) was judged to have reached a value of about 0.2, the reaction was stopped by adding 1 ml 2 M-NaOH and the time was noted. The concentration of *p*-nitrophenol

phosphate in the supernatant was determined by measuring the A_{410} . One unit of enzyme is defined as the amount that hydrolyses one nmol *p*-nitrophenol phosphate in 1 min at 30 °C. Manganese-dependent DNAase was assayed as described by Akriegg (1978). One unit of DNAase is defined as the amount that produces 1 nmol of acid-soluble nucleotides in 20 min at 37 °C. Glucose dehydrogenase was assayed as described by Sadoff (1966). One unit is defined as the amount of enzyme that reduces 1 nmol NAD min⁻¹ at 37 °C. Dipicolinic acid content was measured as described by Janssen *et al.* (1958).

Incidence of heat-resistant spores. Sporulating culture (0.1 ml taken at t_0) was added to 0.9 ml sporulation medium, then heated at 85 °C for 15 min. Samples (0.1 ml) of the treated culture, or a suitable dilution of it, were plated on nutrient agar and colonies were counted after 24 h at 37 °C. Values are given as the number of heat-resistant c.f.u. ml⁻¹ in the original culture.

RESULTS

Directed mutagenesis of the lys-1 region

Twenty-nine sporulation mutants were obtained that showed more than 10% linkage to *lys-1* by transformation. Microscopic examination of nutrient agar cultures after 2 d suggested that 12 were blocked before stage III. The mutations in all 12 showed about 30% linkage to *lys-1*, which is similar to that of other mutations in the *spoIIA* locus (Yudkin & Turley, 1981).

Production of alkaline phosphatase and DNAase in spoIIA mutants

Previous investigations (Waites *et al.*, 1970; Piggot, 1973; Akriegg & Mandelstam, 1978) showed that *spoIIA* mutants do not produce alkaline phosphatase (Pho⁻ phenotype) or DNAase (Nuc⁻ phenotype) during sporulation. However, preliminary screening of the 12 possible *spoIIA* mutants using DNAase detection agar showed that five of them produced DNAase (Nuc⁺) whereas the remainder did not (Nuc⁻). These Nuc⁺ mutants, 560, 561, 562, 563 and 564, and one of the Nuc⁻ strains, 565, were then tested quantitatively for DNAase production during sporulation. Alkaline phosphatase was also measured since production of this enzyme is usually correlated with production of DNAase in sporulation mutants (Akriegg & Mandelstam, 1978).

All five Nuc⁺ mutants produced both DNAase and alkaline phosphatase: the values at t_5 were roughly half of those found for the wild-type. Strain 565 and all of the previously characterized *spoIIA* mutants produced insignificant amounts (<5%) of these enzymes (Table 2). Although the former mutants did not produce the full amounts of alkaline phosphatase and DNAase, they were clearly 'positive' in the qualitative sense and will therefore be referred to as possessing the Pho⁺ Nuc⁺ phenotype. The time-course of production of both enzymes was similar in all Pho⁺ Nuc⁺ mutants to that of the wild-type (see Figs 3 and 4). Generally, alkaline phosphatase activity began to appear after 1.5 h in sporulation medium and DNAase about 1 h later.

These mutants had all been obtained by introducing mutagenized DNA into the same recipient strain and should therefore have been isogenic. It was nevertheless possible that some other mutations producing partial suppression of the Spo⁻ Pho⁻ Nuc⁻ phenotype had been introduced inadvertently into these strains. For this reason the mutations *spo-561* and *spo-562* were transferred by congression with Met⁺ into strain MB75 (*met lys*). The transformants were checked and found to have the same sporulation phenotypes as their parents.

Other phenotypic properties of the mutants

None of the new sporulation mutants produced glucose dehydrogenase or dipicolinic acid (later sporulation marker events) during sporulation (data not shown). Although some of the mutants were oligosporogenous (Table 2), this trait was not correlated with the production of alkaline phosphatase and DNAase since at least one Pho⁻ Nuc⁻ mutant (42.1) was also oligosporogenous.

The Pho⁺ Nuc⁺ mutants differed from the Pho⁻ Nuc⁻ mutants in one further characteristic. It was observed that cultures of Pho⁺ Nuc⁺ mutants tended to lyse in sporulation medium, whereas cultures of Pho⁻ Nuc⁻ strains did not (see Fig. 1 for a typical result). For the first 4 h of incubation no difference was observed between strains 560 (Pho⁺ Nuc⁺), 1.1 (Pho⁻ Nuc⁻) and

Table 2. Phenotypic properties of *spoIIA* mutants

Cultures (15 ml) were induced to sporulate as described in Methods. Samples were taken at intervals for measurement of sporulation marker events.

Strain	Alkaline phosphatase activity at t_5 (units ml ⁻¹)	DNAase activity at t_5 (units ml ⁻¹)	Heat resistance at t_9 (c.f.u. ml ⁻¹)
SG19 (Spo ⁺)	93	1100	Spo ⁺ (2.4×10^8)
560	41	700	High Osp (2.2×10^5)
561	36	560	Low Osp (1.8×10^2)
562	34	370	Low Osp (1.0×10^2)
563	37	580	Low Osp (3.0×10^2)
564	38	640	Low Osp (1.5×10^2)
565	3.8	0	Spo ⁻ ($<5 \times 10^1$)
1.1	3.5	3.0	Spo ⁻ ($<5 \times 10^1$)
37.1	2.8	2.3	Spo ⁻ ($<5 \times 10^1$)
42.1	3.3	4.5	High Osp (1.2×10^5)
50.2	1.0	0	Spo ⁻ ($<5 \times 10^1$)
63.6	1.3	0	Spo ⁻ ($<5 \times 10^1$)
69.4	4.0	3.9	Spo ⁻ ($<5 \times 10^1$)

Fig. 1. Growth during sporulation. SG16 (Pho⁺ Nuc⁺ Spo⁺) (●), 42.1 (Pho⁻ Nuc⁻ Osp) (■) and 560 (Pho⁺ Nuc⁺ Osp) (▲) were induced to sporulate as described in Methods. At intervals the turbidity (A_{600}) of each culture was measured.

the wild-type. After t_4 or t_5 , however, the turbidity of strain 560 began to decline whereas that of strain 1.1 remained relatively constant. The wild-type culture reached a plateau and then showed a renewed increase in turbidity between t_5 and t_9 , probably as a result of the development of refractile spores. In similar experiments all Pho⁺ Nuc⁺ mutants behaved like strain 560, and Pho⁻ Nuc⁻ mutants behaved like strain 1.1 (data not shown).

Mapping of *spoIIA* mutations

The phenotypic properties of the Pho⁺ Nuc⁺ mutants suggested that their mutations lay in a hitherto unrecognized locus distinct from *spoIIA*. Accordingly, three-factor crosses were carried out to determine the positions of the new mutations with respect to *lys-1* and the previously characterized *spoIIA* mutations (Table 3). Recombination indices (Table 4) were calculated (see Methods) and the distances shown on the map (Fig. 2) are given in terms of these. These data showed that the new mutations were interspersed throughout the previously mapped portion of the *spoIIA* locus (Yudkin & Turley, 1981). Liu *et al.* (1982) showed that there are at least two

Table 3. *Three-factor crosses*

Reciprocal three-factor crosses were performed as described in Methods. Lys⁺ transformants were selected and examined for their Spo phenotype

<i>spo</i> mutation in recipient	<i>spo</i> mutation in donor	Proportion of Spo ⁺ among Lys ⁺ transformants	Percentage of Spo ⁺	Probable map order*
<i>spoIIA1</i>	<i>spo-560</i>	11/1350	0.81	<i>lys-1</i> ... <i>spoIIA1</i> ... <i>spo-560</i>
<i>spo-560</i>	<i>spoIIA1</i>	7/2640	0.27	
<i>spoIIA1</i>	<i>spo-561</i>	14/1990	0.70	<i>lys-1</i> ... <i>spoIIA1</i> ... <i>spo-561</i>
<i>spo-561</i>	<i>spoIIA1</i>	5/1560	0.32	
<i>spoIIA1</i>	<i>spo-562</i>	62/1120	5.5	<i>lys-1</i> ... <i>spoIIA1</i> ... <i>spo-562</i>
<i>spo-562</i>	<i>spoIIA1</i>	47/3530	1.3	
<i>spoIIA37</i>	<i>spo-564</i>	15/2150	0.70	<i>lys-1</i> ... <i>spo-564</i> ... <i>spoIIA37</i> †
<i>spo-564</i>	<i>spoIIA37</i>	10/1350	0.74	
<i>spoIIA42</i>	<i>spo-562</i>	10/2660	0.38	<i>lys-1</i> ... <i>spoIIA42</i> ... <i>spo-562</i>
<i>spo-562</i>	<i>spoIIA42</i>	4/1640	0.24	
<i>spoIIA42</i>	<i>spo-565</i>	28/6770	0.41	<i>lys-1</i> ... <i>spoIIA42</i> ... <i>spo-565</i>
<i>spo-565</i>	<i>spoIIA42</i>	9/5190	0.17	
<i>spoIIA69</i>	<i>spo-562</i>	5/6200	0.081	<i>lys-1</i> ... <i>spo-562</i> ... <i>spoIIA69</i>
<i>spo-562</i>	<i>spoIIA69</i>	13/6190	0.21	
<i>spoIIA69</i>	<i>spo-565</i>	6/6160	0.097	<i>lys-1</i> ... <i>spo-565</i> ... <i>spoIIA69</i>
<i>spo-565</i>	<i>spoIIA69</i>	10/3390	0.28	
<i>spo-560</i>	<i>spo-561</i>	12/3000	0.40	<i>lys-1</i> ... <i>spo-561</i> ... <i>spo-560</i>
<i>spo-561</i>	<i>spo-560</i>	54/7030	0.77	
<i>spo-560</i>	<i>spo-562</i>	285/5660	5.0	<i>lys-1</i> ... <i>spo-560</i> ... <i>spo-562</i>
<i>spo-562</i>	<i>spo-560</i>	110/6050	1.8	
<i>spo-560</i>	<i>spo-563</i>	184/6910	2.7	<i>lys-1</i> ... <i>spo-560</i> ... <i>spo-563</i>
<i>spo-563</i>	<i>spo-560</i>	21/1840	1.1	
<i>spo-560</i>	<i>spo-564</i>	109/3420	3.2	<i>lys-1</i> ... <i>spo-560</i> ... <i>spo-564</i>
<i>spo-564</i>	<i>spo-560</i>	12/1220	1.0	
<i>spo-562</i>	<i>spo-564</i>	187/8400	2.2	<i>lys-1</i> ... <i>spo-564</i> ... <i>spo-562</i>
<i>spo-564</i>	<i>spo-562</i>	45/1050	4.3	
<i>spo-562</i>	<i>spo-565</i>	0/18000	0	No detectable recombination
<i>spo-565</i>	<i>spo-562</i>	0/20000	0	
<i>spo-563</i>	<i>spo-564</i>	14/1750	0.80	<i>lys-1</i> ... <i>spo-563</i> ... <i>spo-564</i> †
<i>spo-564</i>	<i>spo-563</i>	37/6060	0.61	

* This was determined as described in Methods.

† The order given is not determinable from the three-factor cross, but is consistent with data from two-factor crosses.

Table 4. *Recombination indices for pairs of mutations in the spoIIA locus*

Recombination indices were determined (see Methods) to obtain a measure of the distance between *spo* mutations.

<i>spo</i> mutation in recipient	<i>spo</i> mutation in donor	Percentage of Spo ⁺ colonies		RI [(b)/(a)]
		(a) <i>spo</i> DNA	(b) <i>spo</i> ⁺ DNA	
<i>spoIIA1</i>	<i>spo-560</i>	0.81 (11/1350)	44 (184/419)	0.020
<i>spoIIA1</i>	<i>spo-561</i>	0.70 (14/1990)	36 (185/512)	0.019
<i>spoIIA1</i>	<i>spo-562</i>	5.5 (62/1120)	44 (92/210)	0.13
<i>spoIIA1</i>	<i>spo-565</i>	4.2 (46/1094)	31 (39/127)	0.14
<i>spoIIA42</i>	<i>spo-562</i>	0.38 (10/2660)	31 (186/588)	0.012
<i>spoIIA42</i>	<i>spo-565</i>	0.41 (28/6770)	31 (186/588)	0.013
<i>spo-560</i>	<i>spoIIA69</i>	7.0 (70/1010)	36 (295/727)	0.19
<i>spo-560</i>	<i>spo-562</i>	5.0 (285/5660)	41 (144/402)	0.12
<i>spo-560</i>	<i>spo-563</i>	2.7 (184/6910)	32 (149/470)	0.080
<i>spo-560</i>	<i>spo-564</i>	3.2 (109/3420)	41 (295/727)	0.078
<i>spo-561</i>	<i>spo-560</i>	0.77 (54/7030)	36 (194/539)	0.021
<i>spo-562</i>	<i>spoIIA69</i>	0.21 (13/6190)	29 (161/530)	0.0072
<i>spo-563</i>	<i>spo-564</i>	0.80 (14/1750)	29 (224/780)	0.028
<i>spo-564</i>	<i>spoIIA37</i>	0.74 (10/1350)	29 (81/282)	0.026
<i>spo-564</i>	<i>spo-562</i>	4.3 (45/1050)	39 (298/774)	0.11
<i>spo-564</i>	<i>spo-565</i>	2.3 (22/960)	24 (99/411)	0.096
<i>spo-565</i>	<i>spoIIA69</i>	0.28 (10/3390)	30 (171/571)	0.0093

Fig. 2. Map of the *spoIIA* locus. The vertical lines show the positions of mutations ordered using the data in Table 3. Those mutations previously mapped (Yudkin & Turley, 1981) are distinguished by asterisks. The map distances shown are the recombination index values from Table 4. Those mutations which could not be separated by recombination are bracketed.

cistrons in the *spoIIA* locus. Thus mutations *spoIIA42*, *spoIIA50* and *spoIIA69*, to the right of the map shown in Fig. 2, were complemented by the plasmid pHM2 carrying a portion of the locus: mutations *spoIIA1*, *spoIIA37* and *spoIIA63* were not. Since mutation *spo-562* appeared to be located in a different cistron from the other mutations having a Pho^+ Nuc^+ phenotype (see Fig. 6) its position was confirmed in the following experiment. Strains 69.4, 562 and 565 were transformed with purified pHM2 DNA (1 μg). Spo^+ transformants were selected and in each case appeared with a frequency of about 10^5 ml^{-1} . In control experiments, spontaneous Spo^+ colonies were not detected (frequency $< 50 \text{ ml}^{-1}$). This result indicated that mutations *spo-562* and *spo-565* both lie within the cloned DNA sequence on pHM2.

Construction of a double mutant

From the data presented it is clear that mutations conferring Pho^+ Nuc^+ and Pho^- Nuc^- phenotypes may lie in either the complemented or non-complemented parts of the locus (see Table 6). Thus, it appears that mutations that are very closely linked and lie in either of two, or possibly more, cistrons can cause either partial or total loss of the ability to produce alkaline phosphatase and DNAase, as well as varying degrees of oligosporogeny.

There are two ways of interpreting these results: firstly that the gene products act sequentially as regulators; secondly that the gene products are subunits of a single regulatory protein. These alternatives might be distinguished by the properties of a double mutant carrying mutations in different *spoIIA* cistrons both of which confer a Pho^+ Nuc^+ Osp phenotype on single mutants.

A double mutant of this type, carrying the mutations *spoIIA561* and *spoIIA562* (one at each end of the map shown in Fig. 2), was isolated from a transformation cross using strain 562.1 as recipient and DNA from strain 561. About 5% of the Spo^- progeny of this cross differed slightly in appearance from the remainder. One of these unusual colonies was isolated (strain 566) and it was shown to be a *spoIIA561/spoIIA562* double mutant as follows. DNA from it was used to transform a series of recipients carrying one or other of the parental mutations to Lys^+ and the progeny were examined for their Spo phenotype (Table 5). No Spo^+ transformants were obtained in crosses with 561.1 (*spoIIA561*) or 562.1 (*spoIIA562*). As a control, strain 564.1 (*spoIIA564*) was treated similarly and it gave a small number of Spo^+ transformants (0.32%). The small proportion of Spo^+ transformants in this cross was expected since *spoIIA564* lies between *spoIIA561* and *spoIIA562* (Fig. 2). In other control crosses (Table 5), DNA from strain 561 (*spoIIA561*) gave Spo^+ transformants with strains 562.1 and 564.1 but not with 561.1, and DNA from strain 562 (*spoIIA562*) gave Spo^+ transformants with strains 561.1 and 564.1 but not with strain 562.1.

Table 5. *Confirmation of the genotype of strain 566*

DNA from strains 561, 562 and 566 was used to transform strains 561.3, 562.3 and 564.3 in turn to Lys⁺. The transformants were examined for their Spo phenotype. The numbers of colonies examined are given in parentheses.

Recipient	Percentage of Spo ⁺ colonies obtained using DNA from:		
	561 (<i>spoIIA561</i>)	562 (<i>spoIIA562</i>)	566 (<i>spoIIA561 spoIIA562</i>)
561.3 (<i>spoIIA561</i>)	0 (1650)	1.9 (377)	0 (2180)
562.3 (<i>spoIIA562</i>)	3.3 (660)	0 (1050)	0 (3280)
564.3 (<i>spoIIA564</i>)	1.3 (1593)	4.3 (1050)	0.32 (10650)

Fig. 3

Fig. 4

Fig. 3. Alkaline phosphatase production by *spoIIA* single and double mutants during sporulation. SG16 (Spo⁺) (●), 561 (*spoIIA561*) (▲), 562 (*spoIIA562*) (■) and 566 (*spoIIA561 spoIIA562*) (○) were induced to sporulate as described in Methods. At intervals samples were taken and assayed for alkaline phosphatase activity.

Fig. 4. DNAase production by *spoIIA* single and double mutants during sporulation. SG16 (Spo⁺) (●), 561 (*spoIIA561*) (▲), 562 (*spoIIA562*) (■) and 566 (*spoIIA561 spoIIA562*) (○) were induced to sporulate as described in Methods. At intervals samples were taken and assayed for DNAase activity.

Fig. 5. Growth of *spoIIA* single and double mutants during sporulation. SG16 (Spo⁺) (●), 561 (*spoIIA561*) (▲), 562 (*spoIIA562*) (■) and 566 (*spoIIA561 spoIIA562*) (○) were induced to sporulate as described in Methods. At intervals the turbidity (A_{600}) of each culture was measured.

Table 6. *Genetic and phenotypic properties of spoIIA mutations*

The upper portion of the Table gives the map locations of the mutations in their linear order (see Fig. 2). Below each mutation the phenotypic characteristics are given using data from Table 2 and Results.

	Non-complementable							Complementable				
	1	561	560	563	564	37	63	42	562	565	69	50
Phosphatase	—	+	+	+	+	—	—	—	+	—	—	—
DNAase	—	+	+	+	+	—	—	—	+	—	—	—
Sporulation	—	Osp	Osp	Osp	Osp	—	—	Osp	Osp	—	—	—
Cell stability	+	—	—	—	—	+	+	+	—	+	+	+

Phenotypic properties of the double mutant

In sporulation experiments (Figs 3 and 4) strain 566 showed a typical $\text{Pho}^- \text{Nuc}^-$ phenotype and it had lost the susceptibility to lysis exhibited by both of its parents and by the other $\text{Pho}^+ \text{Nuc}^+$ strains (Fig. 5). In addition, this strain was asporogenous (< 50 heat resistant c.f.u. ml^{-1} at t_0) unlike its oligosporogenous parents (Table 2). Strain 566 therefore resembled $\text{Pho}^- \text{Nuc}^- \text{Spo}^-$ single mutants rather than either of its parents.

DISCUSSION

For clarity, the genetic and phenotypic properties of the mutants described in this paper have been assembled in a single diagram (Table 6). The portion of the map to the right of mutations *spoIIA37* and *spoIIA63* represents that portion of the locus complemented by the plasmid pHM2 (Liu *et al.*, 1982).

From our results it is readily apparent that mutations within this right-hand gene can produce either the Spo^- or Osp phenotypes. This confirms the earlier observations of Coote (1972*a, b*) that oligosporogeny could be considered as the phenotypic consequence of a leaky mutation in a *spo* gene. More interesting, in the present context, is the fact that mutation *spoIIA562* has the phenotype $\text{Pho}^+ \text{Nuc}^+ \text{Osp Stability}^-$, whereas the closely linked mutations *spoIIA565* and *spoIIA42* generate the phenotype $\text{Pho}^- \text{Nuc}^- \text{Spo}^- \text{Stability}^+$.

In the left-hand sector of the locus one again finds that mutations that are very near to one another may produce sharply contrasting phenotypes (cf. mutations *spoIIA1* and *spoIIA561*).

We can now consider one of the possibilities suggested in the Introduction: that the genes for alkaline phosphatase and DNAase lie on operons that are on the main pathway of spore formation. If that were the case, the partial production of the enzyme should be quite closely correlated with the partial production of spores, i.e. with the degree of oligosporogeny. Clearly, however, this is not so. For instance in strain 562, the synthesis of appreciable amounts of phosphatase and DNAase is associated with a low degree of oligosporogeny (about 100 spores ml^{-1}). By contrast, in strain 42.1 there is no measurable production of either enzyme but the spore formation is about 1000-fold greater (Table 2). This comparison alone shows clearly that the genes for the two enzymes are not on operons of the main pathway. If further support for this contention is required it can be found by comparing the phenotypes of strains 560 and 561. Phosphatase and DNAase are produced to about the same extent by both (the differences in Table 2 are within the limits of day-to-day variation), whereas the incidence of spores differs by a factor of 10^3 .

If it is accepted on this basis that the genes for phosphatase and DNAase are not on operons of the main pathway, the next possibility to consider is that the structural genes for these enzymes may lie together on some other operon and that consequently the two proteins are induced coordinately. There is nothing in Table 2 to contradict this assumption, but it is known from the earlier reports of Akrigg & Mandelstam (1978) that the appearance of phosphatase precedes that of DNAase. Although the intervening period is somewhat variable (40 to 60 min), it is seen quite consistently (Figs 3 and 4). The kinetics of appearance of the two enzymes thus indicate that they are synthesized at distinctly separate times and it is reasonable to conclude that their

structural genes lie in two different operons. Of course the possibility cannot be ruled out that the enzymes are synthesized simultaneously but that the secretion of one of them is delayed for about an hour. However, this seems less likely than the preceding explanation.

We have largely excluded from this discussion, so far, the stability of the cells in sporulation medium. Although it is likely that the lysis of cells in sporulation medium is due to the induction of a lytic enzyme, we have not identified the enzyme and we cannot, therefore, say anything about the kinetics of its appearance. Nevertheless, the data in Table 6 allow us to conclude firstly that closely linked mutations within a single gene can give rise to both negative and positive stability phenotypes and secondly that either phenotype can occur in conjunction with Spo^- or Osp . By the same reasoning as before it can be deduced that the gene concerned is not on an operon of the main sporulation pathway, but it could be on either of the operons containing the genes for phosphatase or DNAase. It cannot consequently be assigned to a fourth operon, though this remains possible.

Coming now to the protein products of the *spoIIA* locus, at least two alternative explanations are possible. If it is assumed that the locus codes for two polypeptides then (a) the polypeptides could act successively, perhaps enzymically, on some substrate that is converted by their action to a functional inducer or derepressor, or (b) the polypeptides could be subunits of a protein functioning as a regulator.

On either assumption a nonsense mutation in either gene should produce the phenotype $Pho^- Nuc^- Spo^-$. In fact, one of the mutations, that in strain 1, was shown by Yudkin & Turley (1981) to be a nonsense mutation and its phenotype is as predicted. Clearly, this is an indeterminate finding.

In an attempt to obtain a more interpretable result, a double mutant was constructed using two parents, 561 and 562, each carrying a mutation in a different gene of the locus. Both parents were of the low Osp type (about $100 \text{ spores ml}^{-1}$) and $Pho^+ Nuc^+$. In the double mutant the spore count was reduced to $< 50 \text{ ml}^{-1}$. This result would be predicted on either hypothesis and is again indeterminate. However, the phenotype was $Pho^- Nuc^-$, neither enzyme having been produced in significant amounts. If the gene products of the cistrons in *spoIIA* were acting consecutively one would have expected the double mutant to produce reasonable amounts of both enzymes. The fact that it does not supports but does not prove the assumption that the two polypeptides specified by the *spoIIA* locus are subunits of a single regulatory protein and do not act successively.

In summary, it is clear that the *spoIIA* locus has a regulatory function, that both its protein products are involved, and that mutations in either can result in contrasting phenotypic effects in at least two, and possibly three, sporulation operons.

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