Use of Temperature-sensitive Mutants to Study Gene Expression of Two Closely Linked Sporulation Loci in *Bacillus subtilis*

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The *spo0B* and *spoIVF* loci are contiguous on the chromosome of *Bacillus subtilis*, so that genes in these loci may be parts of a single polycistronic operon. Temperature-sensitive strains having mutations in these loci were isolated, and temperature-shift experiments were carried out to investigate expression of the genes. The temperature-sensitive periods of *spo0B* mutants extended from the beginning of sporulation until the end of the stage II. The temperature-sensitive periods of *spoIVF* strains were during stage IV of sporulation. Therefore, although the *spo0B* and *spoIVF* loci are contiguous on the chromosome it is unlikely that genes in them are parts of a single polycistronic operon.

INTRODUCTION

In an accompanying paper (Lamont & Mandelstam, 1984), we discussed the possibility that sporulation genes in *Bacillus subtilis* are in polycistronic operons, and that mutations in different genes in a single operon might give blocks at different stages of sporulation. The *spo0B* and *spoIVF* sporulation loci, which lie near to *pheA* on the chromosome, are almost certainly contiguous (Hoch *et al.*, 1978; see also below). They could therefore contain genes which are parts of the same polycistronic operon, as suggested by Hoch *et al.* (1978). It is known that the *spo0B* locus is expressed during vegetative growth and the early stages of sporulation (Brehm *et al.*, 1975; Hirochika *et al.*, 1981), but work on the expression of the *spoIVF* locus has not been reported.

The use of temperature-sensitive mutants to determine the times at which the products of sporulation genes are involved in the process was investigated by Young (1976). We have applied the method to temperature-sensitive strains with mutations in the *spo0B* and *spoIVF* loci. Our results show that while the *spo0B* gene is expressed during vegetative growth and the earliest stages of sporulation, the *spoIVF* locus appears to be expressed much later. It is therefore unlikely that genes in these loci are parts of a single polycistronic operon.

METHODS

Organisms. The strains used are listed in Table 1. Strains 580, 581 and 587 were identified as being temperature-sensitive sporulation mutants following directed mutagenesis (Errington & Mandelstam, 1983) of the *pheA* region of the chromosome, as described in the accompanying paper (Lamont & Mandelstam, 1984).

Growth and sporulation. This was done as described in the accompanying paper (Lamont & Mandelstam, 1984). The temperatures at which cultures were incubated are given in the Figure legends.

Alkaline phosphatase. Samples were removed from cultures at t_5 and were assayed for the presence of alkaline phosphatase by the method of Glenn & Mandelstam (1971).

Measurement of heat-resistance. This was done as described by Lamont & Mandelstam (1984).

Temperature-shift experiments. For 'shift-up' experiments, cultures (70–80 ml) were incubated at 34 °C. Portions (3–5 ml) were removed at regular intervals, put into 100 ml flasks, and incubated at 42 °C. At least 15 min after the

Abbreviation: RI, recombination index.

Table 1. *Organisms*

Strain	Genotype	Sporulation phenotype	Source
168	<i>trpC2</i>	Spo ⁺	Laboratory stock
MB68	<i>pheA12 lys-1</i>	Spo ⁺	Laboratory stock
MB68.1	<i>lys-1</i>	Spo ⁺	Laboratory stock
MB79	<i>pheA12 metC3</i>	Spo ⁺	Laboratory stock
580	<i>spo0B580 lys-1</i>	Spo ^{ts}	Directed mutagenesis of MB68
581	<i>spo0B581 lys-1</i>	Spo ^{ts}	Directed mutagenesis of MB68
587	<i>spoIVF587 lys-1</i>	Spo ^{ts}	Directed mutagenesis of MB68
181	<i>spoIVF181 trpC2</i>	Spo ^{ts}	Laboratory stock
181.3	<i>spoIVF181 lys-1</i>	Spo ^{ts}	Phe ⁺ Spo ^{ts} transformant of strain MB68, donor 181
181.4	<i>spoIVF181 metC3</i>	Spo ^{ts}	Phe ⁺ Spo ^{ts} transformant of strain MB79, donor 181
132	<i>spoIVF132 trpC2</i>	Spo ⁻	Laboratory stock
132.3	<i>spoIVF132 lys-1</i>	Spo ⁻	Laboratory stock
488.1	<i>spo0B136 lys-1</i>	Spo ⁻	Laboratory stock
488.3	<i>spo0B136 purA16</i>	Spo ⁻	Laboratory stock

last sample had been taken, the sample cultures were transferred to 37 °C and incubated overnight, to allow the release of spores from cells. The numbers of heat-resistant spores present in each culture were then measured.

For 'shift-down' experiments, the same method was used except that the large (70–80 ml) cultures were incubated at 42 °C, and the samples (3–5 ml) were incubated at 34 °C. The samples were incubated at 30 °C overnight. Cells in samples which were incubated at 34 °C before t_0 (i.e. while growing in hydrolysed casein medium) were transferred to resuspension medium when the cultures reached appropriate cell densities.

Calculation of the temperature-sensitive periods of mutants. The temperature-sensitive periods of the mutants were calculated using the method of Esposito *et al.* (1970), as described by Young (1976). If f_i is the fraction of total sporulation time when the temperature-sensitive period is initiated, and f_e the time when it ends, then $f_i = t_i \sigma / t_a$ and $f_e = t_e / t_a$, where t_i is the time in hours after resuspension at 42 °C at which a 'shift-down' reduces the number of heat-resistant colony-forming units by 10%, t_e is the time at which 'shift-up' results in the formation of 10% of the maximum number of heat-resistant colony-forming units and t_a (the 'total sporulation time') is the time at which continuous incubation of the mutant at the permissive temperature results in 10% of the maximum number of heat-resistant c.f.u. σ is the ratio for the wild-type of total sporulation time at the permissive temperature to that at the restrictive temperature. When calculating f_i , σ compensates for the fact that sporulation proceeds more slowly at the permissive temperature than at the restrictive temperature. The temperature-sensitive period of each mutant is from f_i to f_e . In calculating f_i and f_e in this way, it is assumed that any particular stage takes the same amount of time in all strains at each incubation temperature.

Transformation. DNA was prepared by the method of Marmur (1961) or by the method of Ward & Zahler (1973). Transformation was carried out, and recombination indices (RI) were calculated, as described in the accompanying paper (Lamont & Mandelstam, 1984). An RI of 0.1, or less, indicates that two mutations probably lie in the same or contiguous genes (Carlton, 1966), and an RI of about 0.3 or less indicates that two mutations may lie in different genes in the same operon (Piggot & Coote, 1976).

RESULTS

Isolation and characterization of temperature-sensitive mutants

Strains 580, 581 and 587 were isolated following directed mutagenesis of the *pheA* region of the *B. subtilis* chromosome. Strains 580 and 581 formed half-length cells during incubation in sporulation medium, a phenotype which is associated with certain *spo0* mutations (Dunn *et al.*, 1976), and failed to form alkaline phosphatase (a stage II event). Values for the co-transformation of these mutations with *pheA* were 74% (209/281) for *spo-580* and 78% (626/801) for *spo-581*. These are in agreement with previously reported values for mutations in the *spo0B* locus (Hoch & Mathews, 1973). The RI values between the newly-identified mutations and a mutation which is known to lie in the *spo0B* locus are shown in Table 2. The values obtained were less than 0.1, and so they indicate that mutations *spo-580* and *spo-581* are likely to lie in the *spo0B* locus.

Strain 181 was isolated by Dr D. Grant (unpublished results) and the *spo* mutation which it carries was found to be 93% linked to *pheA* by PBS1-mediated transduction. Strains 181 and 587

Table 2. *Recombination indices between newly identified spo mutations and known spo mutations*

Strains carrying the newly identified *spo* mutations were transformed with saturating amounts of DNA ($2-8 \mu\text{g ml}^{-1}$) prepared by the method of Marmur (1961) from (i) strain 168 *trpC2* and (ii) strain 488.3 *spo0B136 purA16* or strain 132 *spoIVF trpC2*. [Mutation *spo-136* was assigned to the *spo0B* locus by Hoch & Mathews (1973); mutation *spo-132* was assigned to the *spoIVF* locus by Coote (1972b).] Prototrophic transformants were selected in all crosses. The proportion of Spo^+ recombinants arising from each cross was found after 3-4 d incubation at 37°C . Similar crosses were carried out to determine the RI between *spo0B136* and *spoIVF132*. RI values were calculated by the method of Coote (1972b).

Recipient strain	Donor strain	
	488.3 (<i>spo0B</i>)	132 (<i>spoIVF</i>)
580 <i>spo-580 lys-1</i>	0.08	0.24
581 <i>spo-581 lys-1</i>	0.03	0.23
587 <i>spo-587 lys-1</i>	0.16	0.05
181.4 <i>spo-181 metC3</i>	0.30	0.20
132.3 <i>spoIVF132 lys-1</i>	0.18	
488.1 <i>spo0B136 lys-1</i>		0.21

Table 3. *Effect of temperature on sporulation of Spo⁺ strains*

Strains were induced to sporulate at 34°C and at 42°C by transfer to resuspension medium following growth in casein hydrolysate medium. Samples of each culture were heated at 80°C for 15 min after at least 6 h incubation at 42°C or 12 h at 34°C . They were then diluted and plated onto nutrient agar. The numbers of heat-resistant c.f.u. ml^{-1} were found after overnight incubation at 37°C . The values given are the averages of at least three experiments at each temperature except for strains 181.3 and 181.4 (two experiments). The complete genotypes of the strains are given in Table 1.

Strain	Heat-resistant c.f.u. ml^{-1} after incubation at 34°C (a)	Heat-resistant c.f.u. ml^{-1} after incubation at 42°C (b)	Ratio b/a
MB68.1	1.8×10^8	1.4×10^8	0.78
580 <i>spo0B</i>	1.7×10^8	2.0×10^6	0.012
581 <i>spo0B</i>	6.7×10^7	5.3×10^5	0.008
587 <i>spoIVF</i>	2.9×10^8	1.6×10^6	0.005
181 <i>spoIVF</i>	1.0×10^8	3.0×10^6	0.030
181.3 <i>spoIVF</i>	1.1×10^7	3.5×10^4	0.003
181.4 <i>spoIVF</i>	7.0×10^7	3.5×10^4	0.048

formed wild-type amounts of alkaline phosphatase during sporulation and formed phase-grey prespores, as detected by phase-contrast microscopy. No further prespore development took place in cells of these strains. This phenotype is the same as that associated with mutations in the *spoIVF* locus (Coote, 1972a). Values for the co-transformation of these mutations with *pheA* were 58% (771/1319) for *spo-181* and 70% (98/142) for *spo-587*. These are in agreement with previously reported values for mutations in the *spoIVF* locus (Hranueli *et al.*, 1974). The RI values between the newly identified mutations and a mutation which lies in the *spoIVF* locus are shown in Table 2. They indicate that the new mutations are in the same gene as mutation *spoIVF132*, or in the gene adjacent to it.

On the basis of the phenotypic and genetic criteria described above, mutations *spo-580* and *spo-581* were assigned to the *spo0B* locus, and mutations *spo-587* and *spo-181* to the *spoIVF* locus.

RI values between mutations in the *spo0B* and *spoIVF* loci range between 0.16 and 0.30 (Table 2). These values indicate that the two loci may well be contiguous and could be parts of a single operon (Piggot & Coote, 1976), as has been suggested previously (Hoch *et al.*, 1978).

Sporulation of mutants during incubation at 34°C and at 42°C

The incidences of formation of heat-resistant spores by different strains during incubation at 34°C and at 42°C are shown in Table 3. The total sporulation times of the temperature-sensitive strains at 34°C , and of the Spo^+ strain MB68.1 *lys-1* at 34°C and at 42°C , were found by

Fig. 1. Total sporulation time of strain MB68.1 *Spo*⁺ incubated at 34 °C and at 42 °C. A culture of strain MB68.1 *lys*-1 *Spo*⁺ was grown in casein hydrolysate medium incubated at 34 °C or at 42 °C and was then transferred to sporulation medium. Incubation was continued at the same temperature. Samples were taken at intervals and were assayed for the presence of heat-resistant spores. The maximum values were $1.76 \times 10^8 \text{ ml}^{-1}$ (34 °C) and $1.17 \times 10^8 \text{ ml}^{-1}$ (42 °C). t_a denotes the total sporulation time and is taken as the point at which 10% of the maximum number of heat-resistant spores were present. At 34 °C (○) t_a was 9.25 h and at 42 °C (●) t_a was 5.0 h. σ , the ratio of the total sporulation time of strain MB68.1 at 34 °C to that at 42 °C, was 1.75 (9.25/5.0).

Fig. 2. Effects of temperature-shift on sporulation of strains 580 and 587. (a) 580, (b) 587. ○, 'Shift down': cultures growing at 42 °C in casein hydrolysate medium were transferred to sporulation medium and were incubated at 42 °C. Samples were removed at intervals and incubated at 34 °C. Samples which were moved to 34 °C while growing in casein hydrolysate medium were transferred to sporulation medium when they reached the appropriate cell density, and incubated at 34 °C. The numbers of heat-resistant spores ultimately formed in each sample were measured. The maximum values were $2.20 \times 10^8 \text{ ml}^{-1}$ (580) and $2.17 \times 10^8 \text{ ml}^{-1}$ (587). t_i is the time at which 90% of the maximum number of heat-resistant spores were formed. ●, 'Shift up': cultures growing at 34 °C in casein hydrolysate medium were transferred to sporulation medium and were incubated at 34 °C. Samples were removed at intervals and incubated at 42 °C. The numbers of heat-resistant spores ultimately formed in each sample were measured. The maximum values were $1.98 \times 10^8 \text{ ml}^{-1}$ (580) and $4.00 \times 10^8 \text{ ml}^{-1}$ (587). t_e is the time at which 10% of the maximum number of heat-resistant spores were formed.

Fig. 3. Temperature-sensitive periods of *spo0B* and *spoIVF* strains. The temperature-sensitive periods of the strains used are represented by areas of shading. They were found by calculating the values of f_i (beginning of the temperature-sensitive period) and f_e (end of the temperature-sensitive period) (see Methods and Table 4).

Table 4. Determination of the temperature-sensitive periods of sporulation mutants

Graphs illustrating the derivation of the parameters t_a (total sporulation time), t_i (inhibition time) and t_e (escape time) are shown in Figs 1 and 2. These parameters were found for each strain. The parameters f_i and f_e , which represent the start and the end of the temperature-sensitive periods, were then calculated using the method of Esposito *et al.* (1970), as described in Methods, and with a value for σ of 1.75 (see text and Fig. 1). All values represent the average values of two or three experiments. Values of t_a , t_i and t_e are expressed in h.

Strain	t_a	t_i	t_e	f_i	f_e
580 <i>spo0B lys-1</i>	7.0	-0.5	1.75	-0.07	0.25
581 <i>spo0B lys-1</i>	7.0	-0.5	2.0	-0.07	0.29
587 <i>spoIVF lys-1</i>	6.75	2.25	4.25	0.58	0.63
181 <i>spoIVF trpC2</i>	7.25	2.0	3.75	0.48	0.53
181.3 <i>spoIVF lys-1</i>	8.5	1.75	6.5	0.36	0.76
181.4 <i>spoIVF metC3</i>	7.25	2.0	4.75	0.48	0.66

incubating cultures at the appropriate temperatures in resuspension medium and then taking samples at intervals and testing them for the numbers of heat-resistant spores which were present. The results of experiments with strain MB68.1, and the derivation of the total sporulation time (t_a), are shown in Fig. 1. The total sporulation times of all the strains, found from experiments similar to those shown in Fig. 1, are listed in Table 4. σ , the ratio of the total sporulation time of strain MB68.1 at 34 °C to that at 42 °C, was 1.75. This is similar to the value of 1.70 obtained by Young (1976).

Temperature-sensitive periods of *spo0B* and *spoIVF* strains

The temperature-sensitive periods of the mutants were determined by 'shift-up' and 'shift-down' experiments. The results of experiments with strains 580 and 587 are shown in Fig. 2. The results obtained with all of the strains used are given in Table 4.

The parameters f_i and f_e which represent, respectively, the beginning and end of the temperature-sensitive periods of the different strains, were calculated using the formula of Esposito *et al.* (1970), as described in Methods. The values of f_i and f_e obtained with each strain are given in Table 4, and the temperature-sensitive periods are represented in Fig. 3.

DISCUSSION

Strains 580 *spo0B*, 581 *spo0B*, 181 *spoIVF* and 587 *spoIVF* are temperature-sensitive for sporulation. We have used temperature-shift experiments to determine the temperature-sensitive periods of these strains. The results are shown diagrammatically in Fig. 3.

Strains 580 and 581 had similar temperature-sensitive periods. Any other finding would have been surprising since the *spo0B* locus contains only one complementation group (Hirochika *et al.*, 1981; Ferrari *et al.*, 1982). The frequencies of sporulation of these strains were influenced by the incubation temperature during vegetative growth as well as during sporulation (Fig. 2a and Table 4). This shows that the *spo0B* gene was expressed during vegetative growth, as has been reported previously (Brehm *et al.*, 1975; Hirochika *et al.*, 1981). The temperature-sensitive periods of strains 580 and 581 extended to the end of stage II. Sato *et al.* (quoted in Hirochika *et al.*, 1981) stated that the temperature-sensitive period of a strain carrying a different *spo0B* mutation ends at $t_{1.5}$, which is a similar result if the incubation temperature used by those workers was 37 °C.

The temperature-sensitive periods of the *spoIVF* strains did not begin until after stage III (Fig. 3). The temperature-sensitive periods of strains 587, 181 and 181.4 ended during stages IV–V. These strains are defective in synthesis of the spore cortex, which normally occurs during stage IV, so that the temperature-sensitive periods of these strains coincide with the times at which their phenotypic defects become apparent. Unexpectedly, the temperature-sensitive period of strain 181.3 was found to be longer than those of the other *spoIVF* strains (Fig. 3), and this strain formed significantly fewer heat-resistant spores at both the restrictive and the permissive temperatures (Table 3). These characteristics are presumably related to each other. There is no obvious reason for the low frequency of sporulation and the long temperature-sensitive period of strain 181.3, relative to the parental strain 181. Five other *Spo⁻ Lys⁻* isolates obtained following transformation of strain MB68 by DNA prepared from strain 181 all formed heat-resistant spores with the same frequency as strain 181.3 (results not shown). We have no explanation for the fact that different strains having the same mutation (*spo-181*) gave different results.

The possible effects on the gene products of mutations causing temperature sensitivity, and the relationship of temperature-sensitive periods to the times of gene-expression, are thoroughly discussed by Young (1976). The temperature-sensitive periods of strains carrying mutations in the *spo0B* locus are at the very beginning of sporulation, whereas those of strains carrying mutations in the *spoIVF* locus do not begin until stage IV. The clearly different temperature-sensitive periods show that their protein products function at well-separated stages of sporulation. The fact that the *spo0B* and *spoIVF* loci are adjacent on the chromosome might have indicated that they were different genes in a single polycistronic operon which was expressed during vegetative growth and the early stages of sporulation, and that the *spoIVF* gene-product did not intervene in sporulation until stage IV. This explanation could only be correct if the *spoIVF587* and *spoIVF181* gene-products were able to rapidly re-assume an active conformation after heat-inactivation. If this were not so – and it is very unlikely – the temperature-sensitive periods of the *spoIVF* strains would have been seen to occur during the early stages of sporulation. The alternative explanation, which is much the more likely, is that genes in the *spo0B* and *spoIVF* loci are not parts of a single polycistronic operon and that they are expressed separately during sporulation. Further work, involving cloning of the genes in these loci and the study of their expression during sporulation, would be required to distinguish between these possibilities.

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