Suppression of Asporogeny in *Bacillus subtilis*. Allele-specific Suppression of a Mutation in the *spoIIA* Locus

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From a strain carrying *spoIIA69*, a mutation giving rise to asporogeny, a revertant was isolated which sporulated at about 10^{-2} of the wild-type frequency but in which the time-course of sporulation was much protracted. Genetic analysis of this revertant showed that it retained *spoIIA69* but had acquired a secondary mutation *sas*. *sas* failed to suppress mutations in *spoIID*, *spoIIE* or *spoIIG*; it also failed to suppress another mutation in the *spoIIA* locus. *sas* is extremely closely linked (recombination frequency $\leq 1\%$) with the mutation *spoIIA69* that it specifically suppresses. Strains carrying *sas* alone sporulated at a frequency at least two orders of magnitude below that in the *spoIIA69 sas* double mutant. It is suggested that *spoIIA69* and *sas* lead to compensating amino acid changes in the protein specified by the *spoIIA* locus.

INTRODUCTION

The study of *spo* mutants has made a most valuable contribution to our understanding of sporulation in *Bacillus subtilis*. The fact that *spo* mutations are pleiotropic gives powerful support to the concept that events in sporulation form a dependent sequence (see Mandelstam, 1976), and investigations of the phenotype of mutants blocked at different stages in the sequence have enabled many of these events to be placed in a precise order (Piggot & Coote, 1976). In spite of intensive work, however, the study of *spo* mutants has not so far given much information about the means by which the development of spores is regulated.

There have been several reports of the isolation, from *spo* mutants, of partially revertant strains (work reviewed by Piggot & Coote, 1976: see also Piggot & Taylor, 1977; Trowsdale *et al.*, 1979). These strains had regained certain properties associated with the sporulation sequence, but they were still asporogenous.

We have now begun a search for partially suppressed *spo* mutants (i.e. for strains which, while retaining the *spo* mutation that gave rise to asporogeny, have acquired a suppressor mutation that now permits them to make some spores) in the hope that a study of such suppressors may help to elucidate the control of spore development. In this paper, we report the isolation of a partially suppressed derivative of a strain carrying the mutation *spo-69* in the *spoIIA* locus. We show that the new isolate carries a secondary mutation, very closely linked to *spoIIA69*, but that this suppressor has no effect on any other *spo* mutation with which we have combined it.

METHODS

Organisms. These are described in Table 1.

Media. Growth medium and resuspension medium were as described by Sterlini & Mandelstam (1969). Solid minimal medium was the lactate/glutamate agar of Piggot (1973), but lacking alanine. All of these media were supplemented, when required, with L-cysteine ($20 \mu\text{g ml}^{-1}$), other L-amino acids ($50 \mu\text{g ml}^{-1}$) or uracil ($10 \mu\text{g ml}^{-1}$). In some experiments rifampicin ($2.5 \mu\text{g ml}^{-1}$) or sulphamylamide ($100 \mu\text{g ml}^{-1}$) was used. Nutrient agar was from Oxoid.

Table 1. *Bacillus subtilis* strains

In the methods of construction, recipient $\times$ PBS1(donor) (A⁺) means that the recipient was transduced with phage PBS1 grown on the donor (Takahashi, 1963); A⁺ recombinants were selected and the desired marker was obtained by cotransduction. Recipient $\times$ DNA(donor) (A⁺) means that the recipient was transformed with DNA purified from the donor; A⁺ recombinants were selected and the desired marker was obtained by cotransformation. Recipient $\times$ donor (*b* by congression with A⁺) means that the recipient was transformed with a stationary phase culture of the donor (Ephrati-Elizur, 1968); A⁺ recombinants were selected and the desired marker was obtained by congression.

Laboratory stock strains		
Strain	Genotype	Previous designation and reference
<i>spo</i> ⁺ strains		
BD112	<i>cysA</i>	
GSY254	<i>lys-1 trpC2</i>	
MB10	<i>pyrD1</i>	
MB35	<i>hisA aroB2 leuA rfm</i>	
168 sul	<i>trpC2 Sul</i> ^R	
<i>spo</i> mutants		
1	<i>trpC2 spoIIA1</i>	E1 (Piggot, 1973)
20	<i>trpC2 spoIIE20</i>	N25 (Waites <i>et al.</i> , 1970)
55	<i>trpC2 spoIIG55</i>	NG12.12 (Piggot, 1973)
68	<i>trpC2 spoIID68</i>	NG17.29 (Coote & Mandelstam, 1973)
69.2	<i>metC3 spoIIA69</i>	NG18.6 (Piggot, 1973)
Constructed strains		
Strain	Genotype	Construction
MY2001	<i>hisA pyrD1 aroB2 rfm</i>	MB35 $\times$ MB10 (<i>pyrD1</i> by congression with Leu ⁺)
MY2002	<i>hisA pyrD1 lys-1 rfm</i>	MY2001 $\times$ PBS1(GSY254) (Aro ⁺)
MY2003	<i>hisA pyrD1 cysA rfm</i>	MY2002 $\times$ BD112 (<i>cysA</i> by congression with Lys ⁺)
MY2007	<i>metC3 spoIIA69 sas-1</i>	see Results
MY2009	<i>metC3 spoIIA69 sas-2</i>	see Results
MY2010	<i>hisA pyrD1 rfm sas-1</i>	MY2002 $\times$ DNA(MY2007) (Lys ⁺)
MY2011	<i>lys-1 pyrD1 rfm sas-1</i>	MY2010 $\times$ GSY254 (<i>lys-1</i> by congression with His ⁺)
MY2012	<i>hisA pyrD1 rfm sas-2</i>	MY2002 $\times$ DNA(MY2009) (Lys ⁺)
MY2013	<i>lys-1 pyrD1 rfm sas-2</i>	MY2012 $\times$ GSY254 (<i>lys-1</i> by congression with His ⁺)
MY2014	<i>lys-1 hisA cysA rfm</i>	MY2003 $\times$ GSY254 (<i>lys-1</i> by congression with Ura ⁺)
MY2016	<i>lys-1 hisA rfm Sul</i> ^R	MY2014 $\times$ PBS1(168 sul) (Cys ⁺)
MY2018	<i>hisA pyrD1 rfm</i>	MY2002 $\times$ DNA(MY2007) (Lys ⁺)
MY2019	<i>hisA pyrD1 rfm spoIIA69 sas-1</i>	MY2002 $\times$ DNA(MY2007) (Lys ⁺)
MY2020	<i>hisA pyrD1 rfm spoIIA69 sas-2</i>	MY2002 $\times$ DNA(MY2009) (Lys ⁺)
MY2000.01H	<i>hisA pyrD1 rfm spoIIA1</i>	MY2002 $\times$ 1 (<i>spoIIA1</i> by congression with Lys ⁺)
MY2000.01L	<i>lys-1 pyrD1 rfm spoIIA1</i>	MY2002 $\times$ 1 (<i>spoIIA1</i> by congression with His ⁺)
MY2000.20H	<i>hisA pyrD1 rfm spoIIE20</i>	MY2003 $\times$ PBS1(20) (Cys ⁺)
MY2000.20L	<i>lys-1 pyrD1 rfm spoIIE20</i>	MY2000.20H $\times$ GSY254 (<i>lys-1</i> by congression with His ⁺)
MY2000.55	<i>lys-1 pyrD1 rfm spoIIG55</i>	MY2002 $\times$ 55 (<i>spoIIG55</i> by congression with His ⁺)
MY2000.68	<i>lys-1 pyrD1 rfm spoIID68</i>	MY2002 $\times$ 68 (<i>spoIID68</i> by congression with His ⁺)
MY2000.69	<i>lys-1 pyrD1 rfm spoIIA69</i>	MY2002 $\times$ 69.2 (<i>spoIIA69</i> by congression with His ⁺)

Sporulation experiments. Bacteria were transferred from growth medium to resuspension medium as described by Sterlini & Mandelstam (1969). After incubation with shaking (for 15 h at 37 °C, except where otherwise mentioned), a sample was taken and divided into two portions. One was diluted and plated on nutrient agar; the other was first heated for 30 min at 85 °C, then diluted if necessary and plated on nutrient agar. Colonies were counted after 18 to 24 h incubation at 37 °C. The incidence of spores is given as the number of heat-resistant spores per colony-forming unit (c.f.u.).

Transformation. Transformation with stationary phase cultures of the donor was by the technique of Ephrati-Elizur (1968). Transformation with a limiting concentration (0.1 µg ml⁻¹) of purified DNA [made by the method of Marmur (1961)] was by the technique of Ayad & Barker (1969). Recombinants were selected on minimal agar with appropriate supplements. Plates were incubated at 37 °C for 3 d and then at room temperature for 2 to 4 d. Spo⁺ (sporogenous), Spo⁻ (asporogenous) and various Osp (oligosporogenous) colonies could then be readily distinguished. Spo⁺ colonies were brown and opaque, Spo⁻ colonies were colourless and almost transparent, and Osp strains had various intermediate appearances that depended

on the degree of oligosporogeny. The assignment of colonies to sporulation phenotypes was frequently confirmed by studying their resistance to chloroform vapour. Colonies that had grown on minimal agar in glass plates were replica-plated to nutrient agar, exposed to chloroform vapour at 60 °C for 30 min, and then again replica-plated to nutrient agar. The two replicas were compared after incubation at 30 °C for 16 to 24 h. Replicas of *Spo*⁺ colonies grew equally well on the two plates, *Spo*⁻ colonies were completely absent from the second plate, and *Osp* colonies gave various intermediate results depending on the degree of oligosporogeny.

Mutagenesis. (i) With ethyl methanesulphonate. To 5 ml bacteria growing exponentially in growth medium at 37 °C (about 200 µg dry wt ml⁻¹) was added 0.1 ml ethyl methanesulphonate (Sigma). After 15 min incubation with shaking at 37 °C, the bacteria were recovered by centrifugation, washed in warm resuspension medium, and resuspended in 20 ml warm brain heart infusion broth. The culture was shaken at 37 °C until the bacterial density reached about 250 µg dry wt ml⁻¹, and the cells were then sedimented by centrifugation and transferred to resuspension medium.

(ii) With ultraviolet irradiation. Cells growing exponentially in 5 ml growth medium at 37 °C (about 200 µg dry wt ml⁻¹) were collected by centrifugation, transferred to 2 ml resuspension medium and irradiated with ultraviolet light (about 3 J m⁻² s⁻¹) for 2 min. The irradiated suspension was added to 20 ml warm brain heart infusion broth in a foil-covered flask and incubated with shaking at 37 °C. When the bacterial density reached 250 µg dry wt ml⁻¹, the cells were sedimented by centrifugation and transferred to resuspension medium.

RESULTS

Isolation of partial revertants of strain 69.2

Strain 69.2 carries *spoIIA69*, a mutation that blocks sporulation at stage II (Piggot, 1973). When cells harvested from an exponentially growing culture of strain 69.2 were shaken in sporulation salts for about 15 h at 37 °C and then heat-treated samples were plated out, colonies arose at a frequency of about 2 per 10⁹ c.f.u. All of these appeared to be fully *Spo*⁺ revertants, as judged by the appearance of the colonies and by the incidence of spores in a few chosen at random.

In an attempt to find partial revertants, strain 69.2 was treated with ethyl methanesulphonate. Resuspension cultures of mutagenized strain 69.2 contained about eight times as many heat-resistant spores as untreated cultures. About one-quarter of the colonies arising from these spores appeared to be *Spo*⁺, but the remainder had the less opaque appearance characteristic of *Osp* (oligosporogenous) mutants. One of these was picked and purified (MY2007).

In order to isolate another partial revertant, a culture of strain 69.2 was treated with ultraviolet radiation. Resuspension cultures of ultraviolet-treated strain 69.2 contained scarcely any more heat-resistant spores than untreated cultures; however, we did recover one colony of *Osp* appearance from a heat-treated resuspension culture of irradiated 69.2. This was named MY2009.

On microscopic examination about 8 h after resuspending MY2007 or MY2009 in sporulation salts, no phase-bright spores were found among more than 1000 cells examined, and only about 10⁻⁴ of the c.f.u. were heat-resistant. In the wild-type, by contrast, most cells contained phase-bright, heat-resistant spores at that time. After 16 h incubation in resuspension medium, occasional phase-bright spores could be seen in cultures of MY2007 or MY2009, and about 10⁻² of the c.f.u. were heat-resistant, compared with 50 to 80 % in the wild-type. (This point is examined in more detail below.) Strains MY2007 and MY2009 were thus classified as oligosporogenous.

Genetic analysis of strains MY2007 and MY2009

There are two obvious possible explanations for the oligosporogenous phenotype of MY2007 and MY2009. Either the mutation *spoIIA69* has partially reverted, or it has remained unchanged but has been partially suppressed by a second mutation. In the latter case it should be possible to separate the two mutations.

Table 2. *Summary of results of transformation experiments*

DNA purified from *lys*⁺ donors was used at limiting concentration to transform *lys* recipients, and the number of *Lys*⁺ transformants falling into each of four sporulation phenotypes was determined for each cross. 'High Osp' strains gave about 1 spore per 10² c.f.u., 'Low Osp' strains about 1 spore per 10⁴ to 10⁶ c.f.u., and 'Spo⁻' strains less than 1 spore per 10⁸ c.f.u., after 18 h at 37 °C in re-suspension medium. The percentages given are illustrative; figures such as 70 % and 30 % are not intended to represent the precise result of any particular experiment. Only the sporulation genotypes are given; other markers, omitted in this Table for clarity, are listed in Table 1.

Donor strain	Recipient strain	Total <i>Lys</i> ⁺ examined	Approximate frequency of phenotypic classes among <i>Lys</i> ⁺ transformants (%)			
			Spo ⁺	Spo ⁻	High Osp	Low Osp
69.2 (<i>spoIIA69</i>)	MY2002 (<i>spo</i> ⁺)	750	70	30	0	0
MY2007 (<i>spoIIA69 sas-1</i>)	MY2002 (<i>spo</i> ⁺)	1720	70	0	30	0.1
MY2009 (<i>spoIIA69 sas-2</i>)	MY2002 (<i>spo</i> ⁺)	1660	70	0	30	0.3
168 Sul ^R (<i>spo</i> ⁺)	MY2011 (<i>sas-1</i>)	400	30	0	0	70
168 Sul ^R (<i>spo</i> ⁺)	MY2013 (<i>sas-2</i>)	530	30	0	0	70
MY2010 (<i>sas-1</i>)	MY2000.69 (<i>spoIIA69</i>)	12000	0	70	0.3	30
MY2012 (<i>sas-2</i>)	MY2000.69 (<i>spoIIA69</i>)	10000	0	70	0.3	30
69.2 (<i>spoIIA69</i>)	MY2000.69 (<i>spoIIA69</i>)	15000	0	100	0	0
MY2007 (<i>spoIIA69 sas-1</i>)	MY2000.69 (<i>spoIIA69</i>)	15000	0	70	30	0
MY2010 (<i>sas-1</i>)	MY2000.01L (<i>spoIIA1</i>)	260	4	67	0	29
MY2012 (<i>sas-2</i>)	MY2000.01L (<i>spoIIA1</i>)	420	5	67	0	28
MY2000.01H (<i>spoIIA1</i>)	MY2011 (<i>sas-1</i>)	1860	0.7	30	0	69
MY2000.01H (<i>spoIIA1</i>)	MY2013 (<i>sas-2</i>)	840	1.5	30	0	69
MY2012 (<i>sas-2</i>)	MY2011 (<i>sas-1</i>)	15400	0	0	0	100
MY2010 (<i>sas-1</i>)	MY2013 (<i>sas-2</i>)	17400	0	0	0	100

spoIIA69 is known to be linked to *lys* by transformation (Piggot, 1973). When DNA purified from strain 69.2 was used to transform strain MY2002 (*spo*⁺ *lys*), about one-third (27 to 36 %) of the *Lys*⁺ transformants were Spo⁻. When purified DNA from strain MY2007 was used to transform strain MY2002, about two-thirds of the *Lys*⁺ transformants were Spo⁺ and one-third resembled MY2007; however, two out of 1720 transformants had a more translucent, colourless appearance. Similarly, when strain MY2002 was transformed with DNA from MY2009, five out of 1660 transformants were more translucent and colourless than is characteristic of strain MY2009. (The results of these and subsequent transformations are summarized in Table 2.)

We initially suspected that this minority class of seven transformants carried *spoIIA69* only, separated by recombination from a suppressing mutation, but subsequent work disproved this belief. When streaked on plates of minimal agar and incubated for 3 d at 37 °C, these transformants appeared more opaque than strain MY2000.69 (an isogenic strain carrying *spoIIA69*) but less opaque than MY2007 or MY2009, which suggested that they represented a class that produced spores at a low frequency, but higher than that of strains carrying *spoIIA69* alone. In fact, when tested in resuspension experiments (in which MY2007 and MY2009 gave heat-resistant spores at a frequency of about 1 per 10² c.f.u. and MY2000.69 at a frequency of less than 1 per 10⁸ c.f.u.) all seven of these transformants gave heat-resistant spores at a frequency of 1 per 10⁴ to 10⁶ c.f.u. These spores themselves gave rise to similar oligosporogenous populations. Repetition of these experiments showed that such variability of about two orders of magnitude in the frequency of appearance of spores was characteristic of these strains: there was no evidence that the seven transformants belonged to more than one class. The sporulation phenotype of the strains just described, and of others mentioned below, is given in Table 3.

Our interpretation of these facts is as follows. Strains MY2007 and MY2009 arose by the

Table 3. Incidence of spores in resuspension cultures of mutant strains

The numbers of heat-resistant spores and of total c.f.u. were determined after 18 h at 37 °C in resuspension medium. The incidence of spores is given to the nearest power of 10.

Strain	Sporulation genotype	Approximate incidence of spores
69.2	<i>spoIIA69</i>	$< 10^{-8}$
MY2007	<i>spoIIA69 sas-1</i>	10^{-2*}
MY2009	<i>spoIIA69 sas-2</i>	10^{-2*}
MY2010, MY2011	<i>sas-1</i>	10^{-4} to 10^{-6}
MY2012, MY2013	<i>sas-2</i>	10^{-4} to 10^{-6}
MY2019	<i>spoIIA69 sas-1</i>	10^{-2*}
MY2020	<i>spoIIA69 sas-2</i>	10^{-2*}
MY2000.01H, MY2000.01L	<i>spoIIA1</i>	10^{-8}
MY2000.69	<i>spoIIA69</i>	$< 10^{-8}$

* The time-course of sporulation in these strains was very protracted.

acquisition of secondary mutations by strain 69.2. These secondary mutations partially suppress the asporogenous mutation *spoIIA69*. We call the secondary mutation in MY2007 *sas-1* (for suppression of asporogeny) and that in MY2009 *sas-2*. Mutations *sas-1* and *sas-2* are both very closely linked to *spoIIA69*, but they can be separated from it at a low frequency. The rare class among the Lys⁺ transformants produced when MY2002 was transformed with DNA from MY2007 or MY2009 contain only *sas*, separated from *spoIIA69*; because recombinants carrying only *spoIIA69* were not recovered in these crosses, *sas-1* and *sas-2* probably lie between *spoIIA69* and *lys*. Strains carrying *sas-1* (MY2010) or *sas-2* (MY2012) unaccompanied by any other *spo* mutation sporulate at a rather low but variable frequency; hence *sas-1* and *sas-2* are themselves *spo* mutations.

Phenotypes of strains carrying *sas* mutations

When cultures of MY2000.69 that had been incubated in resuspension medium for several hours at 37 °C were examined by light microscopy, most of the cells had an abortively disporic appearance. This phenotype is characteristic of strains carrying mutations at the *spoIIA* locus (Piggot & Coote, 1976). Resuspension cultures of the *sas* mutants MY2010 and MY2012 were indistinguishable in microscopic appearance from MY2000.69, which tends to bear out our conclusion that the *sas* mutations are, in reality, mutations in *spoIIA*, very closely linked to *spoIIA69*. Cells taken from resuspension cultures of the double mutants MY2019 and MY2020 (Table 1) were again indistinguishable.

Although the incidence of heat-resistant spores in resuspension cultures of MY2010 (*sas-1*) and MY2012 (*sas-2*) was quite variable, the incidence in corresponding cultures of MY2019 (*spoIIA69 sas-1*) and MY2020 (*spoIIA69 sas-2*) was more constant. On several occasions the number of heat-resistant spores finally obtained in resuspension cultures of MY2019 and MY2020 was between 5 per 10³ c.f.u. and 2 per 10² c.f.u. However, the time-course of sporulation in these strains was very protracted (Fig. 1). Heat-resistant spores appeared in a resuspension culture of MY2020 several hours later than in a similar *spo*⁺ culture (strain MY2019 was indistinguishable from MY2020). By contrast, the time-course of appearance of heat-resistant spores in MY2010 (*sas-1*) and MY2012 (*sas-2*) resembled that of the *spo*⁺ strain MY2018.

We wondered whether strains MY2019 and MY2020 might be temperature-sensitive for sporulation, but found that the number of spores finally obtained from these strains was no different at 30, 37 or 41 °C.

Fig. 1. Time-course of appearance of heat-resistant spores in strains MY2012, MY2018 and MY2020. Cells from exponentially growing cultures of MY2012 (*sas-2*) (□), MY2018 (*spo*⁺) (○) and MY2020 (*spoIIA69 sas-2*) (●) were resuspended in sporulation medium. At intervals, samples were taken and the numbers of heat-resistant spores and of total c.f.u. were found. The incidence of heat-resistant spores is plotted as a percentage of the incidence 18 h after resuspension.

Genetic analysis of *sas* mutations

To test our conclusion that *sas-1* and *sas-2* lie in *spoIIA*, we transformed MY2011 (*lys sas-1*) and MY2013 (*lys sas-2*) with DNA from a wild-type. Among the Lys⁺ transformants about one-third (25 to 37 %) were Spo⁺.

We have suggested that *sas-1* and *sas-2* are mutations that are closely linked to *spoIIA69* and partially suppress the asporogeny imposed by the latter, and that MY2010 and MY2012 carry *sas* mutations unaccompanied by other *spo* mutations. If these suggestions are correct, it should be possible to cross MY2000.69 with MY2010 or MY2012 and recover recombinants that resemble the original isolates MY2007 and MY2009.

We transformed MY2000.69 with DNA purified from MY2010. Out of about 12000 Lys⁺ transformants, 33 appeared substantially more opaque and pigmented than the remainder, although readily distinguishable, both by the appearance of the colonies and by their sensitivity to chloroform vapour, from Spo⁺ strains. We chose six of these at random and tested their frequency of sporulation in a resuspension experiment. After 18 h in resuspension medium the number of heat-resistant spores in the six cultures ranged from 4 to 15 per 10³ c.f.u. This frequency of spores is very similar to that in MY2019, which (if our interpretation is correct) has the same sporulation genotype (*spoIIA69 sas-1*).

In a similar experiment, we recovered 27 pigmented colonies from about 10000 Lys⁺ recombinants when we transformed MY2000.69 with DNA from MY2012. In five of these chosen at random the frequency of heat-resistant spores after 18 h in resuspension medium was 5 to 10 per 10³ c.f.u.

The fact that *spoIIA69 sas* double mutants were recovered in these experiments, but *spo*⁺ recombinants were not, supports our belief that the order of the markers is *spo-69*. . . *sas* . . . *lys*. Given this order, *spo*⁺ recombinants could only have been produced in these transformations by quadruple crossovers.

The results can be used to obtain the recombination frequency between *spo-69* and *sas*. When we transformed MY2002 with the DNA from MY2010, 67 % of the Lys⁺ transformants were Spo⁺. Hence, although 12000 Lys⁺ recombinants were obtained when we trans-

formed MY2000.69 with the same DNA preparation, in 8000 of these the *sas-1* marker will not have been available for recombination with *spoIIA69* in the recipient. Of the remaining 4000 transformants, 33 were *spo-69 sas-1* double mutants; thus, the recombination frequency between the markers is 0.8 %. A similar calculation gives a recombination frequency of 0.7 % between *spo-69* and *sas-2*.

In control experiments, we transformed MY2000.69 with DNA from 69.2 or from MY2007. Out of more than 15000 Lys⁺ transformants from each of these crosses, none was Spo⁺ (see Table 2).

Effect of sas mutations on strains carrying mutations in other spoII loci

To see whether *sas-1* and *sas-2* suppressed other *spoII* mutations, rather than *spoIIA69* specifically, *lys* strains carrying *spoIID68*, *spoIIE20* or *spoIIG55* (see Table 1) were each transformed with DNA from MY2010 or MY2012. About one-third of the Lys⁺ transformants in such experiments carried *sas*, so if *sas* mutations suppressed the other *spo* markers one would have expected one-third of these transformants to show a Spo⁺ (or at least Osp) phenotype. In fact, out of several hundred recombinants from each transformation more than 99 % remained Spo⁻, as shown both by the absence of pigment and by their extreme sensitivity to chloroform vapour.

sas mutations do not suppress spoIIA1

We wanted to determine whether *sas* mutations were capable of suppressing a mutation in *spoIIA* other than *spoIIA69*. When MY2000.01L (*spoIIA1 lys*) was transformed with DNA from MY2010 (*sas-1 lys*⁺), about two-thirds of the Lys⁺ transformants resembled strains carrying *spoIIA1* (Spo⁻) and one-third resembled MY2010 (Osp), but 10 out of 260 Lys⁺ transformants were more pigmented than the latter (Table 2). If *sas-1* did in fact suppress *spoIIA1*, one would have expected that among these pigmented colonies there would have been some that carried the two mutations *spoIIA* and *sas-1*. However, this argument is sound only if the order of the markers is *spoIIA1*. . *sas-1*. . *lys*. If the order of the markers were *sas-1*. . *spoIIA1*. . *lys*, then the large majority of pigmented colonies in the above cross would be *spo*⁺, even if the double mutant *spoIIA1 sas-1* were pigmented. We therefore made the reciprocal cross by transforming MY2011 (*sas-1 lys*) with DNA purified from MY2000.01H (*spoIIA1 lys*⁺). Out of 1860 Lys⁺ transformants, 14 were more pigmented than MY2011; 10 of these were picked at random and purified.

We were now sure that, whatever the order of markers, among the 20 pigmented colonies picked from the two reciprocal crosses there would be several that carried both *spoIIA1* and *sas-1*, if this double mutant was more pigmented than strains carrying *sas-1* alone.

All of these 20 pigmented recombinants seemed, by their appearance and their resistance to chloroform vapour, to be Spo⁺, but, to be sure that none of them carried both *sas-1* and *spoIIA1*, each of them was used as a donor in transformation by the technique of Ephrati-Elizur (1968). The recipient was MY2016 (*lys hisA* Sul^R), and Lys⁺ recombinants were selected on minimal plates containing histidine and sulphanilamide. If in such a cross the donor carried the two *spo* mutations *spoIIA1* and *sas-1*, then these mutations (which, as the above results show, recombine quite readily) would sometimes be separated. The result would be that a few per cent of the Lys⁺ transformants would be of Spo⁻ or Osp appearance.

We examined at least 500 Lys⁺ transformants from each of the 20 experiments, but in no case was there evidence that the donor contained a *spo* mutation linked to *lys*. (In 15 of the 20 cases all of the transformants were Spo⁺; in the remaining five cases about 10⁻³ of all the Lys⁺ transformants were Spo⁻, but when these were analysed they all resembled strains carrying *spoO* mutations. In no case was the *spo* mutation linked by transformation to *lys*; we suspect that a very few transformants had adventitiously acquired *spoO* mutations.) This entire experiment was repeated by making the two analogous reciprocal crosses between

strains carrying *spoIIA1* and strains carrying *sas-2* and picking pigmented colonies; the results were similar (Table 2).

We concluded that all the pigmented colonies which emerged from crosses between *spoIIA1* and *sas* were *spo*⁺, and therefore that the doubly mutant recombinants *spoIIA1 sas* were not pigmented. Since in these recombination experiments we had been careful to pick all colonies that were more pigmented than strains carrying *sas* alone (and suppressed strains carrying *spoIIA69* together with *sas* could easily be recognized as more pigmented than those carrying *sas* alone), it appeared that *sas* mutations did not suppress *spoIIA1*.

The results of these experiments were used to derive a probable order of the markers *spoIIA1*, *sas-1* and *lys*. With *sas-1 lys*⁺ as donor and *spoIIA1 lys* as recipient, 10 out of 260 *lys*⁺ transformants (3.8 %) were *spo*⁺, but in the reciprocal cross with *spoIIA1 lys*⁺ as donor and *sas-1 lys* as recipient, only 14 out of 1860 *lys*⁺ transformants (0.75 %) were *spo*⁺. If we assume that in the latter case *spo*⁺ recombinants had to be generated by quadruple cross-overs, the order is *sas-1 . . spo-1 . . lys*. The results of the crosses between *sas-2* and *spoIIA1* similarly suggest the order *sas-2 . . spo-1 . . lys* (Table 2).

Attempts to recombine sas-1 and sas-2

Although MY2007 (*spoIIA69 sas-1*) and MY2009 (*spoIIA69 sas-2*) were isolated independently, they were extremely similar both in their genetic behaviour and in their phenotypic effects and it seemed possible that they were, in fact, the same mutation. If they were different, one might have been able to recombine them to generate *spo*⁺.

We transformed MY2011 (*sas-1 lys*) with DNA from MY2012 (*sas-2 lys*⁺) and obtained 15400 *Lys*⁺ recombinants, and we transformed MY2013 (*sas-2 lys*) with DNA from MY2010 (*sas-1 lys*⁺) and obtained 17400 *Lys*⁺ recombinants. All these recombinants were carefully examined; every one retained the *Osp* phenotype of the parents. We therefore suspect that *sas-1* and *sas-2* are identical.

DISCUSSION

We interpret our results as follows. *sas* is a mutation in *spoIIA* which, when unaccompanied by *spoIIA69*, renders strains oligosporogenous. Such strains have, in resuspension cultures, the typical appearance of *spoIIA* mutants. *sas* does not suppress mutations in three other *spo* loci that we have tested [one of which immediately precedes *spoIIA* in the dependent sequence (Piggot & Coote, 1976)], and it does not suppress *spoIIA1*. Had *sas* been discovered during a routine search for *spo* mutations it would have seemed a run-of-the-mill *spoIIA* mutation.

Coote (1972) has produced evidence suggesting that a mutation leading to oligosporogeny can occur in the same gene as a mutation leading to asporogeny. Our results point to the same conclusion: *spoIIA69* and *sas* are very closely linked (recombination frequency $\leq 1\%$), and although it is naturally possible that the mutations are near the ends of contiguous genes it seems more likely that they lie in the same gene. Moreover, we have shown that the probable order of mutations is *spo-69 . . sas . . spo-1 . . lys*, which would place a mutation giving oligosporogeny between two mutations giving asporogeny.

What is remarkable about *sas* is that it partially suppresses *spoIIA69*, a mutation which renders strains asporogenous. One might also say, with some justification, that *spoIIA69* suppresses *sas*: the frequency of spores produced in resuspension cultures of *spoIIA69 sas* double mutants is at least two orders of magnitude higher than in mutants containing *sas* alone. This fact seems important in our understanding of how *sas* works.

We have considered a model in which *spoIIA69* and *sas* are both promoter mutations; when both mutations are present promoter activity is higher than with either alone. However, the combination of *spoIIA69* and *sas*, although it ultimately leads to the production of more spores than are found in the single mutants, renders sporulation unusually pro-

tracted; moreover, *spoIIA1* produces a phenotype indistinguishable from that of *spoIIA69* mutants (unpublished results), and yet the recombination frequency between *sas* and *spoIIA* seems much too high for *spoIIA1* also to lie in the same promoter.

A more likely possibility is that *spoIIA69* is a chain-terminating mutation and *sas* a mutation that reinitiates translation (Grodzicker & Zipser, 1968). Here again, however, the sporulation properties of strains carrying the separate mutations or the two together, although not incompatible with such a model, are not what one would have predicted from it. Moreover, *spoIIA69*, although a point mutation, is not suppressed by the nonsense suppressor *su⁺3* (unpublished results). We have also established that *sas-1* does not suppress the amber mutations *metB5* or *purB6* (results not shown).

The explanation that we favour is that *spoIIA* encodes a protein that is essential for sporulation, that *spoIIA69* produces an amino acid replacement that abolishes activity, and that *sas*, when present alone, produces a replacement that renders the protein very weakly active. However, the two amino acid changes compensate, with the result that a strain carrying both mutations sporulates at a higher frequency than strains carrying either alone. [Compensating amino acid changes are well known in other systems (Helinski & Yanofsky, 1963)]. To verify this explanation one would need to isolate the product of the *spoIIA* gene.

Whether this model is correct or not, it seems clear that each of three different mutant forms of *spoIIA* leads to a characteristic sporulation phenotype: *spoIIA69* alone gives asporogeny; *sas* alone gives low oligosporogeny (10^{-6} to 10^{-4}); *spoIIA69* and *sas* together give high oligosporogeny (about 10^{-2}) with sluggish production of spores. These results suggest the possibility that *spoIIA* may have an important role in the regulation of sporulation. Further study of the mutants described here, and in particular a detailed investigation of the temporal sequence of events during sporulation of *spoIIA69 sas* double mutants (whose delayed sporulation is not immediately explicable in terms of any of the models we have mentioned), might throw some light on the control of spore development.

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