Mapping of Six Mutations in the *spoIIA* Locus of *Bacillus subtilis* and Studies of Their Response to a Nonsense Suppressor

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Six mutations in the *spoIIA* locus have been mapped by transformation crosses. The Recombination Index between mutations at opposite ends of the locus was about 0.17; all six mutations are likely to be allelic. A mutation leading to oligosporogeny was found to be closely linked on both sides to mutations leading to asporogeny. For two pairs of mutants, crosses between the members of each pair yielded no wild-type recombinants, but the mutations were found not to be identical. Another of the mutations was found to be partly suppressed by the general nonsense suppressor *su*⁺3, and is therefore presumably a chain-terminating mutation.

INTRODUCTION

Mutations leading to asporogeny occur at many genetic loci in *Bacillus subtilis* (Piggot & Coote, 1976). A recent report from this laboratory showed that the asporogenous mutation *spo-69* in the *spoIIA* locus could be specifically suppressed by a very closely linked mutation, *sas-1* (Yudkin & Turley, 1980). The properties of strains carrying *sas-1* or the combination *spoIIA69 sas-1* suggested that the product of *spoIIA* might have an important function in regulating spore development in *B. subtilis*. We now present a map of six mutations in *spoIIA* and an account of their response to the general nonsense suppressor *su*⁺3. The map shows, *inter alia*, that a mutation that leads to oligosporogeny lies between, and is most probably in the same gene as, mutations leading to asporogeny. The results of the experiments with *su*⁺3 indicate that one of the six mutations tested is nonsense-suppressible.

METHODS

Organisms. *Bacillus subtilis* strain 168 (*spo*⁺ *trpC2*) was used as the wild-type. Strain MY2010 (*hisA pyrD1 rfm sas-1*) has been described by Yudkin & Turley (1980). The *spo* strains used, from the collection of J. Mandelstam, were six whose mutations have been assigned to the *spoIIA* locus by Piggot & Coote (1976). (A seventh, which formed spores in resuspension medium at an incidence of about 10⁻⁴ of wild-type, was not studied). The six are listed in Table 1; a previous description of them is that of Piggot (1973), who has given reasons for believing that each strain carries a single *spo* mutation. From each strain the *spo* mutation was transferred to strain MY2002 (*hisA pyrD1 lys-1 rfm*) by the technique of Ephrati-Elizur (1968); selection was for His⁺ and *spo* derivatives were obtained by congression. All these have the genotype *pyrD1 lys-1 rfm spoIIA*, and are called MY2000.01, MY2000.37, etc., where the number after the point indicates the *spo* allele. Each *spo* mutation was also transferred to a strain (MY2021) that contains the two suppressible mutations *metB5* and *purB6* (which are not linked by transformation), in addition to the *lys-1* marker. Transfer in this case was by co-transformation (by means of purified DNA) with Lys⁺, and the resulting strains are called MP1, MP37, etc. Into this set of strains the nonsense suppressor *su*⁺3 (Georgopoulos, 1969) was inserted by using DNA purified from DT57.2 (*leu metB5 purB6 su*⁺3) to transform each of the MP strains to Met⁺ and Pur⁺ simultaneously. The resulting strains are called MP1.3, MP37.3, etc. (the 3 after the point indicating the nonsense suppressor). Although each strain of this last set is prototrophic, it was shown to carry a *met* mutation by using it as a donor by the technique of Ephrati-Elizur (1968) to transform MY2002 to Lys⁺, and showing that some of the progeny were Met⁻.

Media. Growth medium, resuspension medium and solid minimal medium have been described previously (Yudkin & Turley, 1980). Nutrient agar for selecting *Spo*⁺ colonies was made according to the recipe of Schaeffer *et al.* (1965).

Mapping experiments. The procedure for transformation with 0.1 µg DNA ml⁻¹ has been described previously (Yudkin & Turley, 1980). Selection for *Lys*⁺ was on minimal agar supplemented with uracil, and *Spo*⁺ colonies were readily distinguished from *Spo*⁻ colonies by their appearance (Yudkin & Turley, 1980). In selecting for *Spo*⁺, the transformation mixture was spread on nutrient agar in glass plates and incubated at 37 °C for 20 h. Chloroform (3 ml) was added to the lid of each plate, the agar-containing dish was inverted over the lid, and the plate was incubated at 50 °C until the chloroform had evaporated. After incubation at 37 °C for a further 3 d, the deeply pigmented *Spo*⁺ colonies could readily be seen against the lawn of killed bacteria. In some experiments, these *Spo*⁺ colonies were tested for their *Lys* phenotype in the following way. The colonies were transferred with sterile toothpicks to minimal agar supplemented with lysine and uracil in glass plates. The resulting patches were incubated at 37 °C for 3 d, exposed to chloroform vapour as described above, and then replicated to minimal agar with uracil alone and to minimal agar with lysine and uracil. The replicas were compared after 20 to 24 h at 37 °C, and could be scored as *Lys*⁺ or *Lys*⁻ unequivocally.

The results of these experiments were used to establish both the distances between pairs of *spo* mutations and the order of the mutations relative to the *lys* marker. As a measure of the genetic distance between the *spo* mutation (here designated *spo1*) in the recipient and the *spo* mutation (designated *spo2*) in the donor, we used the Recombination Index (RI):

$$\frac{[\text{No. of } Spo^+ \text{ transformants (spo2 DNA as donor)}]/[\text{No. of } Lys^+ \text{ transformants (spo2 DNA as donor)}]}{[\text{No. of } Spo^+ \text{ transformants (spo}^+ \text{ DNA as donor)}]/[\text{No. of } Lys^+ \text{ transformants (spo}^+ \text{ DNA as donor)}]}$$

To determine the order of markers, we did reciprocal crosses between strains carrying different *spo* alleles and made use of *lys* as a linked marker. Suppose that the order of the mutations is *spo1*...*spo2*...*lys*. If DNA from a *spo1 lys*⁺ strain is used to transform a *spo2 lys* recipient and selection is made for *Spo*⁺, then the ratio (No. of *Spo*⁺ *Lys*⁺ transformants)/(No. of *Spo*⁺ transformants) will be approximately equal to the co-transformation frequency between *spoIIA* and *lys*; on the other hand, if DNA from a *spo2 lys*⁺ strain is used to transform a *spo1 lys* recipient and selection is made for *Spo*⁺, the ratio (No. of *Spo*⁺ *Lys*⁺ transformants)/(No. of *Spo*⁺ transformants) will be expected to be much lower than the co-transformation frequency between *spoIIA* and *lys*⁺, since a quadruple crossover is necessary to introduce *lys*⁺.

Hence, the results of the reciprocal crosses *spo1 lys*⁺ × *spo2 lys* and *spo2 lys*⁺ × *spo1 lys* should indicate the order of the mutations.

If from the above crosses selection is made for *Lys*⁺ rather than for *Spo*⁺, a study of the number of *Lys*⁺ transformants that are also *Spo*⁺ will corroborate the map order. If *spo1 lys*⁺ is the donor and *spo2 lys* the recipient, among the *Lys*⁺ transformants *Spo*⁺ can be produced by a double crossover; but if *spo2 lys*⁺ is the donor and *spo1 lys* the recipient, the formation of *Spo*⁺ recombinants among the *Lys*⁺ transformants requires a quadruple crossover. Hence, if reciprocal crosses are made and *Lys*⁺ transformants are selected, the fraction of *Spo*⁺ recombinants will be expected to be much larger in the first of these crosses than in the second. (In practice, the numerical value of this fraction will depend on the linkage between *spoIIA* and *lys*, which will differ slightly from one DNA preparation to another; to take account of this difference we divided the crude results by a factor that represents the co-transformation frequency determined experimentally for each DNA preparation. The numbers given in Table 3 are the corrected values.)

Incidence of sporulation. The technique for determining the number of spores after incubation in resuspension medium at 37 °C has been described previously (Yudkin & Turley, 1980).

RESULTS

Genetic mapping of *spoIIA* mutations

DNA was prepared from the *spo*⁺ *lys*⁺ strain 168 and from each of the six mutants listed in Table 1, and used at limiting concentration to transform *lys* derivatives carrying each

Table 1. *Bacillus subtilis spo* strains used

Strain	Genotype	Previous designation (Piggot, 1973)
1	<i>spoIIA1 trpC2</i>	E1
37	<i>spoIIA37 trpC2</i>	NG1.82
42	<i>spoIIA42 trpC2</i>	NG6.13
50	<i>spoIIA50 trpC2</i>	NG11.2
63	<i>spoIIA63 trpC2</i>	NG16.2
69	<i>spoIIA69 trpC2</i>	NG18.6

Table 2. Results of mapping experiments with selection for *Spo*⁺

DNA purified from the *lys*⁺ *spo*⁺ strain 168 and from *lys*⁺ derivatives of each *spo* strain was used to transform *lys* derivatives of each *spo* strain. Selection was for *Spo*⁺ or for *Lys*⁺, and the Recombination Index for each cross was calculated as described in Methods. The Recombination Indices given are the means of two determinations (or of three determinations for those values marked with an asterisk). For each cross, a number of *Spo*⁺ recombinants were tested for their *Lys* phenotype. The way in which the order of mutations was obtained is described in Methods.

<i>spo</i> strains A B	A donor:B recipient			B donor:A recipient			Probable order
	Recombination Index	No. examined for <i>Lys</i> phenotype	% <i>Lys</i> ⁺	Recombination Index	No. examined for <i>Lys</i> phenotype	% <i>Lys</i> ⁺	
1 37	0.11	89	11	0.07	89	33	37 ... 1 ... <i>lys</i>
1 42	0.17*	47	6	0.15*	84	42	42 ... 1 ... <i>lys</i>
1 50	0.21	41	5	0.18	86	50	50 ... 1 ... <i>lys</i>
1 63	0.10	68	12	0.10	85	41	63 ... 1 ... <i>lys</i>
1 69	0.16*	83	11	0.14*	85	39	69 ... 1 ... <i>lys</i>
37 42	0.06	40	10	0.11*	85	43	42 ... 37 ... <i>lys</i>
37 50	0.11	41	10	0.13	88	44	50 ... 37 ... <i>lys</i>
37 63	<0.001			<0.001			
37 69	0.10	44	7	0.13	88	33	69 ... 37 ... <i>lys</i>
42 50	0.05	63	6	0.04	48	38	50 ... 42 ... <i>lys</i>
42 63	0.09	69	62	0.08	45	4	42 ... 63 ... <i>lys</i>
42 69	0.04	93	17	0.04	58	24	
50 63	0.07	69	46	0.08	75	5	50 ... 63 ... <i>lys</i>
50 69	<0.001			<0.001			
63 69	0.10	80	10	0.09	55	49	69 ... 63 ... <i>lys</i>

mutation. From the numbers of *Spo*⁺ and of *Lys*⁺ recombinants, RI values were calculated as described in Methods (Table 2). *Spo*⁺ recombinants were obtained in all crosses except the two pairs *spo*-50 × *spo*-69 and *spo*-37 × *spo*-63. Table 2 also lists the fraction of *Lys*⁺ recombinants among the *Spo*⁺ progeny of each cross: in nearly all cases this fraction was much larger for one of the members of a reciprocal cross than for the other.

In all of the crosses except those between *spo*-50 and *spo*-69 and those between *spo*-37 and *spo*-63, a certain fraction of the *Lys*⁺ transformants had recombined the *spo* markers and become *Spo*⁺. These fractions (corrected as described in Methods) are listed in Table 3: again, one of the members of a reciprocal cross almost always gave a much larger fraction of *Spo*⁺ recombinants among the *Lys*⁺ transformants than the other.

Non-identity of *spo*-50 and *spo*-69

spo-69 has the unusual property of being suppressible by *sas*-I, a mutation that lies extremely close to *spo*-69 but on the *lys* side of it (Yudkin & Turley, 1980). Since the above results had suggested that *spo*-50 and *spo*-69 could not recombine to give *Spo*⁺, it was possible that the two mutations were identical, in which case *spo*-50 too would have been

Table 3. *Results of mapping experiments with selection for Lys⁺*

Lys⁺ recombinants were selected from the crosses described in the legend to Table 2 and examined for their *Spo* phenotype as previously described (Yudkin & Turley, 1980). Each cross was done at least twice. The way in which the order of mutations was obtained is described in Methods.

<i>spo</i> strains		A donor:B recipient		B donor:A recipient		Probable order
A	B	No. examined for <i>Spo</i> phenotype	% <i>Spo</i> ⁺	No. examined for <i>Spo</i> phenotype	% <i>Spo</i> ⁺	
1	37	3690	2.6	1410	12.2	37 ... 1 ... <i>lys</i>
1	42	3520	3.9	990	20.6	42 ... 1 ... <i>lys</i>
1	50	1060	3.3	2580	25.3	50 ... 1 ... <i>lys</i>
1	63	3090	2.1	1060	8.8	63 ... 1 ... <i>lys</i>
1	69	1890	2.1	1010	18.2	69 ... 1 ... <i>lys</i>
37	42	5340	2.8	4820	14.2	42 ... 37 ... <i>lys</i>
37	50	1950	1.4	6670	12.0	50 ... 37 ... <i>lys</i>
37	63	3000	<0.1	9000	<0.1	
37	69	4830	2.3	4770	10.0	69 ... 37 ... <i>lys</i>
42	50	1940	2.2	7720	5.6	50 ... 42 ... <i>lys</i>
42	63	5800	6.5	4710	3.2	42 ... 63 ... <i>lys</i>
42	69	2740	1.7	1850	1.8	
50	63	6070	7.7	2650	0.7	50 ... 63 ... <i>lys</i>
50	69	4120	<0.1	1100	<0.1	
63	69	5400	1.3	2610	7.0	69 ... 63 ... <i>lys</i>

suppressible by *sas-1*. However, when DNA from MY2010 (*sas-1 lys⁺*) was used to transform MY2000.50, none of 45 000 *Lys⁺* transformants had the characteristic, partially pigmented phenotype associated with the combination *spo-69 sas-1*, whereas in a transformation of MY2000.69 done in parallel with the same DNA preparation, some 6 of every 1000 *Lys⁺* transformants were partially pigmented, a result similar to that obtained by Yudkin & Turley (1980).

Order of spo-42 and sas-1

The RI values between *spo-69* and *spo-42* presented in Table 2 are about fourfold higher than that previously established between *spo-69* and *sas-1* (Yudkin & Turley, 1980). The present and previous results taken together suggested the order *spo-69 ... sas-1 ... spo-42 ... lys*. To confirm this order, we transformed the *lys* derivative of *spo-42* with DNA prepared from the *lys⁺ sas-1* strain, MY2010, and selected *Spo⁺* recombinants. Of these, 38% were *Lys⁺*. It was not possible to perform the reciprocal experiment since the incidence of spores in strains carrying *sas-1* is so high that it is impracticable to select *Spo⁺* recombinants with such strains as recipients. However, the high frequency of *Lys⁺* recombinants obtained with the *lys⁺ sas-1* strain as donor and the *lys spo-42* strain as recipient is consistent with the order *sas-1 ... spo-42 ... lys*. In two experiments the RI between *sas-1* and *spo-42* was 0.03, which again is consistent with *sas-1* being very close to and on the *lys* side of *spo-69*.

Response of the spoIIA mutations to nonsense suppression

As is described in detail in Methods, we prepared two isogenic sets of strains, MP1, MP37, etc. and MP1.3, MP37.3, etc. The six strains in the first set each contain one of the *spo* alleles (*spo-1*, *spo-37*, etc.); the six strains in the second set contain the same alleles and each carries in addition the nonsense suppressor *su⁺3*.

The incidence of spores in resuspension cultures of these 12 strains, and of the two isogenic *spo⁺* parents, is given in Table 4. The nonsense suppressor *su⁺3* clearly had little effect on sporulation in the *spo⁺* strains or in five of the mutants, but with *spo-1* the presence of *su⁺3*

Table 4. Incidence of spores in suppressing and non-suppressing strains

The numbers of viable cells and of heat-resistant spores were determined as previously described (Yudkin & Turley, 1980) after 18 h incubation in resuspension medium. The incidence of spores is defined as the number of heat-resistant spores per unit volume of culture divided by the number of viable cells in the same volume. The values quoted are the mean of two determinations. Each pair of strains is isogenic except in respect of the *su*⁺3 marker.

<i>spo</i> genotype	Incidence of spores	
	Strain without suppressor	Strain with <i>su</i> ⁺ 3
<i>spo</i> ⁺	5×10^{-1}	6×10^{-1}
<i>spo-1</i>	1×10^{-7}	5×10^{-3}
<i>spo-37</i>	$<10^{-8}$	$<10^{-8}$
<i>spo-42</i>	4×10^{-5}	12×10^{-5}
<i>spo-50</i>	$<10^{-8}$	$<10^{-8}$
<i>spo-63</i>	1×10^{-8}	1×10^{-7}
<i>spo-69</i>	$<10^{-8}$	$<10^{-8}$

Fig. 1. Map of the *spoIIA* locus. The positions of the mutations are marked by vertical lines. The numbers immediately above the horizontal lines give the RI values between the pairs of mutations to which the arrowheads are pointing. The number closest to each arrowhead shows the RI obtained when the strain carrying that mutation was used as the recipient. Each number is the mean of two or three determinations.

increased the incidence of spores by several orders of magnitude. This dramatic effect of the nonsense suppressor suggests that the product of the *spoIIA* locus – or at least of that part of the locus in which *spo-1* occurs – is a polypeptide; a similar conclusion about the region of the *spoIIA* locus in which *spo-69* and *sas-1* occur was derived, from different lines of evidence, by Yudkin & Turley (1980).

DISCUSSION

The results presented in Tables 2 and 3 allow the mutations to be ordered unequivocally (with the exception of the two pairs [*spo-50*, *spo-69*] and [*spo-37*, *spo-63*], which are further discussed below). All the results are consistent with the order shown in Fig. 1.

The RI values given in Table 2 are also reasonably self-consistent. The RI between [*spo-50*, *spo-69*] and *spo-42* is about 0.04, between *spo-42* and [*spo-37*, *spo-63*] about 0.09, and between [*spo-37*, *spo-63*] and *spo-1* about 0.10. The RI between mutations at opposite ends of the locus (i.e. between [*spo-50*, *spo-69*] and *spo-1*) is about 0.17. Is this RI consistent with all the mutations being in a region that encodes a single polypeptide? Carlton (1966) found that the RI between mutations at opposite ends of the *trpB* gene was about 0.2; this gene codes for the β -subunit of tryptophan synthase, whose (monomeric) molecular weight is 41 000 (Hoch, 1973), which is by no means exceptionally large among polypeptides. There is therefore no reason to assign mutations to different genes because they are separated by an RI of 0.17. Moreover, all of the mutations mapped here produce a similar, very characteristic phenotype when the strains are incubated in resuspension medium (Piggot, 1973), so once again there is no compelling reason to believe that *spoIIA* encodes more than one polypeptide.

We have been unable to detect reversion of *spo-63* in any of several experiments in which, on each occasion, more than 10^9 cells were plated (unpublished results). The remaining five mutations, including *spo-37*, revert to *spo*⁺ at a frequency of about 10^{-8} to 10^{-7} . Since *spo-37* and *spo-63* cannot recombine to give *spo*⁺ (see Table 2), it seems likely that *spo-63* is a small deletion that covers *spo-37*.

Although the reversion frequency of all the mutations other than *spo-63* suggests that they are probably point mutations, we were unable to select Spo⁺ recombinants at a frequency greater than that due to reversion in the crosses of *spo-50* with *spo-69*, no matter which strain was donor and which recipient. Moreover, no Spo⁺ recombinants could be detected among several thousand Lys⁺ transformants from the same crosses. These results suggest that the same nucleotide is mutated in the two strains. On the other hand, *spo-50* and *spo-69* are demonstrably different in their response to the suppressor *sas-1*. [Piggot (1973) also failed to detect recombination to *spo*⁺ in the crosses *spo-37* $\times$ *spo-63* and *spo-50* $\times$ *spo-69*.]

It is remarkable that, out of six mutations isolated at random in the *spoIIA* locus, two should apparently affect the same nucleotide. One possible explanation is that the protein product of *spoIIA* is extremely tolerant of amino acid changes, with the result that only a very small fraction of the large number of missense mutations in *spoIIA* produced by mutagenesis would have a Spo⁻ phenotype. However, mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (which produced five of the mutations studied here) would be expected to yield not only missense but also nonsense mutations, and most nonsense mutations would give a Spo⁻ phenotype irrespective of their precise location. Thus the coincidence of two mutations is not readily explicable in terms of peculiarities of the structure of the protein product of *spoIIA*, but is more likely to be due to the existence of a mutational 'hot-spot' at the level of DNA. Further experiments in mutagenizing the region would confirm the presence of such a 'hot-spot'.

The results presented here have implications for our understanding of mutations that lead to oligosporogeny. Coote (1972) obtained results suggesting that oligosporogenous mutations could occur in genes in which asporogenous mutations were known. The finding of Yudkin & Turley (1980) that the oligosporogenous mutation *sas-1* mapped extremely close to the asporogenous mutation *spo-69* provided support for that view, and the present results show that the oligosporogenous mutation *spo-42* is closely linked on one side to the asporogenous mutations *spo-50*, *spo-69*, and on the other side to the asporogenous mutations *spo-37*, *spo-63*; all five of these are likely to be allelic.

The results with the suppressible mutation *spo-1* provide still stronger support for the view that there is no rigorous distinction between asporogenous and oligosporogenous mutations. *spo-1* is suppressed by *su*⁺₃, which inserts an amino acid in response to a nonsense codon (Camacho *et al.*, 1974). One may presume that *spo-1*, which was produced by mutagenesis with ethyl methanesulphonate (Piggot, 1973) and which reverts (see above), is due to a single base change. Chain termination at this point renders the strain asporogenous, whereas the substitution of the amino acid inserted by *su*⁺₃ for the wild-type amino acid at the same

position renders it oligosporogenous (see Table 4). Had the original mutation yielded the codon for the *su*⁺3 amino acid, the mutant strain would have been classified as oligosporogenous; it was only by chance that the mutation, in fact, yielded a nonsense codon.

su⁺3 is about 10% efficient in inserting an amino acid in response to nonsense encoded in phage DNA (Camacho *et al.*, 1974). However, the incidence of spores in a *spo-1 su*⁺3 strain was only 1% of that in the isogenic *spo*⁺ strain (see Table 4). Perhaps the amino acid inserted by *su*⁺3 so distorts the protein product of *spoIIA* as to render it barely functional; alternatively, the distortion may be only slight, but because of the intricate nature of the interactions needed for sporulation a small change in one protein could seriously affect the whole developmental sequence. The final product of the sequence – the completed spore – is so remote from the expression of a single *spo* locus that we cannot use the incidence of spores as a quantitative measure of that expression.

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