Sporulation in *Bacillus subtilis*. Genetic Analysis of Oligosporogenous Mutants

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(Accepted for publication 23 December 1971)

SUMMARY

Mutations in 30 oligosporogenous (Osp) mutants of *Bacillus subtilis* have been mapped by transduction with phage PBS-1. The sporulation phenotype of these mutants had previously been characterized biochemically and morphologically. The mutations are situated in areas of the genome previously shown to be occupied by asporogenous (Sp⁻) mutations. Data from reciprocal transformation between Osp and Sp⁻ mutations of indistinguishable phenotype suggest that one of the Osp mutations and at least one of the Sp⁻ mutations may be located within the same gene.

INTRODUCTION

In a previous paper the correlation of biochemical events with morphological changes was shown to be the same in oligosporogenous (Osp) and asporogenous (Sp⁻) mutants of *Bacillus subtilis* (Coote, 1972). Only Osp mutants with normal growth in a number of media were selected so as to avoid, as far as was possible, mutants having defective sporulation because of damage to a vegetative function (Spizizen, 1965; Bott & Davidoff-Abelson, 1966; Fortnagel & Freese, 1968). Over half of these Osp mutants exhibited a phenotype previously identified with the Sp⁻ character. This suggested that instead of Osp mutants being considered as a distinct class of sporulation mutant, for example, as regulatory mutants (Hanson, Peterson & Yousten, 1970), the Osp and Sp⁻ states might, in some instances at least, be alternative expressions of mutation within a single gene. This would have to be shown by mapping, and the only evidence on this point which seems so far to have been presented was by Rouyard, Ionesco & Schaeffer (1967) whose data from reciprocal transformation between an Osp mutant and a Sp⁻ mutant blocked at stage II of sporulation indicated that the two mutations may have been located within the same gene.

In this paper I report the mapping by phage PBS-1 mediated transduction of the thirty Osp mutants previously described (Coote, 1972). Also included is an analysis by transformation of six mutants (two Osp and four Sp⁻) exhibiting a similar sporulation phenotype and all linked by transduction to the *phe-12* marker. The Osp mutations are located in the same areas of the genome as Sp⁻ mutations. In addition evidence is given to indicate that an Osp mutation and a Sp⁻ mutation may be located within the same gene.

METHODS

Organisms. *Bacillus subtilis* MARBURG 168 (*trpC*) was used which, although it is an auxotroph, sporulates normally and is referred to as the wild-type. The Osp mutants were derived from this strain as described previously (Coote, 1972). A prototrophic MARBURG

Table 1. *Strains of Bacillus subtilis used for the transduction analysis*

Mutant	Genotype	Origin
GSY254	<i>lys-1, trpC2</i>	C. Anagnostopoulos
GSY289	<i>ura-1, trpC2</i>	C. Anagnostopoulos
GSY334	<i>leu-8</i>	C. Anagnostopoulos
GSY342	<i>metB5</i>	C. Anagnostopoulos
GSY1020	<i>purB6, hisB2</i>	C. Anagnostopoulos
GSY1021	<i>purA16, hisB2</i>	C. Anagnostopoulos
BD112	<i>cysA14</i>	D. Dubnau
BD163	<i>hisA1, argC4</i>	D. Dubnau
BD40	<i>phe-12, argA3</i>	D. Dubnau
BD70	<i>metA3, trpC2</i>	D. Dubnau
BD111	<i>thr-5, trpC2, cysB3</i>	D. Dubnau
MB2	<i>lys-1, hisB2</i>	P. Piggot
MB3	<i>phe-12</i>	P. Piggot
MB8	<i>hisA1</i>	P. Piggot

strain of *B. subtilis* was obtained from Dr S. R. Ayad. The auxotrophic strains used for the genetic analysis are listed in Table 1.

Terminology. Stages O to VI are the generally accepted stages of spore formation and have been described previously (Coote, 1972). Stages O and I were not distinguished in electron micrographs and mutants of these types have been grouped together as stage O mutants.

Media. PAB was antibiotic assay medium no. 3 (Difco, Detroit, U.S.A.) BHIB medium was 2.5% Brain Heart Infusion Broth (Oxoid, London) plus 0.5% yeast extract (Difco). Glutamate minimal medium and lactate-glutamate minimal agar plates were prepared as described previously (Coote, 1972). PTM and TM media were media I and II as described by Ayad & Barker (1969).

Preparation of transducing lysates. The method used was essentially that of Karamata & Gross (1970). Donor cells were grown overnight at 30 °C in PAB, checked to ensure that they were motile and then diluted into 4 flasks containing BHIB medium to give 5×10^6 cells/ml. The cells were infected with 0.1 ml PBS-1 stock lysate at different times (0, 30, 60 and 90 min) following the dilution in BHIB. The cultures were incubated with shaking for 8 to 10 h at 37 °C and then allowed to lyse overnight at room temperature without aeration. The bacterial debris was removed by centrifugation and the supernatants stored at 4 °C in sterile vials containing a few drops of chloroform. The vials were well shaken and left for 1 day before use to ensure that all bacteria had been killed. Originally, lysates were treated with DNase before shaking with chloroform, but this was later omitted because no transforming activity has been detected under the conditions used for transduction. Transducing activities of the lysates prepared at the different times varied. Transducing activity is unrelated to the active phage titre (Karamata & Gross, 1970).

Transduction. Recipient cells were grown in BHIB medium at 37 °C until late log phase (0.5 mg dry wt/ml). If the cells were motile 1 ml of the suspension was added to 0.1 ml of a lysate and incubated at 37 °C with shaking for 30 min. The cells were then centrifuged, resuspended in an equal volume of glutamate minimal medium and 0.1 ml of this suspension spread on a lactate-glutamate minimal agar plate. The sterility of the lysate was tested on nutrient agar (Oxoid) and reversion of the recipient bacteria was tested by plating 0.1 ml of a suspension not mixed with phage, but otherwise treated in an identical manner. When the recipient was a double auxotroph the requirement not being selected for was added to the plates before the addition of the cell suspension.

Transformation. DNA was extracted by the 4-aminosalicylate-phenol method of Kelly &

Pritchard (1965). The DNA fibres were collected by winding on to a glass rod, dipped in 95 % (v/v) ethanol, and redissolved in 5 ml of a solution of NaCl (10 mM) plus EDTA (1 mM). The solution was stored at 4 °C overnight and the following day the concentration of saline was increased to 0.1 M. The DNA was stored frozen in small volumes until used. It was not treated with RNase. The transformation procedure was performed according to Ayad & Barker (1969). Recipients were grown overnight at 37 °C in PTM medium. They were then diluted with the same medium to approximately 0.05 mg dry wt/ml and shaken vigorously at 37 °C until late log phase (0.5 mg dry wt/ml). Five ml of the culture was added to 18 ml of warmed TM medium which lacked the amino acid being selected for. The cells were shaken gently for 40 to 50 min, two 1 ml samples then removed and one of them added to DNA. The cells were shaken for a further 1 h at 37 °C and 0.1 ml of each suspension plated directly on to lactate–glutamate minimal agar. Sterility of the DNA was checked by spreading a drop on to nutrient agar. All transformations were performed using saturating concentrations of DNA (2 µg/ml).

Selection and screening of recombinants. Generally, for transduction, lysates resulting from the infection of the Osp strain with phage were used to transduce an auxotrophic sporulating strain to prototrophy. Recipient strains were chosen having auxotrophic requirements which between them covered the genetic map (see Dubnau, 1970). Both prototrophic transductants and transformants were isolated on lactate–glutamate minimal agar and left for several days to allow development of the pigment associated with sporulation in this strain (Iichinska, 1960; Schaeffer & Ionesco, 1960). Colonies were then scored as Sp⁺ (brown) or Osp (white or light brown). Oligosporogenous transductants and transformants were examined in the phase-contrast microscope to ensure, at least in the case of mutants forming feebly refractile spores, that they had the phenotype of the original mutant and in any case to ensure that they were still Osp in character.

Criteria of linkage. The chromosomal location of the Osp mutations was determined by measuring the co-transduction of the Osp characters with auxotrophic markers. Percentage recombination was the percentage of prototrophic transductants which inherited the unselected Osp marker subtracted from 100 and this figure was used as a rough measure of the distance between two markers. Three-factor crosses in the *lys* region were done using lysates from Osp, *lys*⁺, *trpC* as donor with Sp⁺, *lys*-1, *trp*⁺ as recipient (MB2). *Lys*⁺ transductants were selected and scored as Osp or Sp⁺. They were then tested on lactate–glutamate minimal agar lacking tryptophan to determine the number of *trpC* recombinants. The position of the unknown marker, Osp, with respect to markers of known location, *lys* and *trp*, was determined by analysing the distribution of recombinants from the cross.

For transformation, the linkage relationship between two mutations was determined by two-point crosses and calculated by the method of Lacks & Hotchkiss (1960). Oligosporogenous or asporogenous mutants under investigation, together with the parent wild-type strain, were initially transformed to prototrophy using DNA prepared from the *trp*⁺ strain of *Bacillus subtilis*. DNA was then prepared from each prototrophic mutant and the prototrophic derivative of the parent strain. Any one mutant DNA preparation along with the wild-type DNA preparation were each used in separate experiments to transform a *trpC* mutant strain to prototrophy. *Trp*⁺ transformants from both crosses were selected on lactate–glutamate minimal agar lacking tryptophan and after incubation at 37 °C for 2 to 3 days any Sp⁺ recombinants were clearly distinguishable among the *trp*⁺ transformants. At the saturating concentration of DNA which was used, wild-type DNA and mutant recipient normally gave between 5 and 10% Sp⁺ colonies among the selected *trp*⁺ transformants, i.e. one in 10 to 20 cells transformed to *trp*⁺ were able to take up a second piece

Fig. 1. The distribution of markers on the *Bacillus subtilis* genome according to Dubnau (1970). The numbers are percentage recombination obtained from two-factor transduction crosses and are taken from a previous paper (Dubnau *et al.* 1967).

Fig. 2. Map of the positions of Osp mutations obtained by two-factor transduction crosses. The numbers are percentage recombination and are the average of at least two separate experiments in which 100 to 1000 colonies were scored for each cross. The absence of a number between an auxotrophic marker and a sporulation marker indicates that no linkage was detected. The Roman numerals are the stages of sporulation at which the mutants were blocked and Abn means abnormal development (see Coote, 1972).

Fig. 3. Map of the positions of Osp mutations obtained by two and three-factor transduction crosses. For further details see Fig. 2 and Table 2.

Table 2. *Orientation of Osp mutations with respect to the lys-I and trpC markers by means of three-factor crosses*

Donor genotype	Recipient genotype	Recombinants		Implied order
		Type	No.	
Y13, <i>trpC2</i>	<i>lys-I, (hisB2)</i>	<i>lys⁺Y13trp⁺</i>	63	Y13, <i>lys, trp</i>
		<i>lys⁺Y13trp⁻</i>	15	
		<i>lys⁺Sp⁺trp⁺</i>	24	
		<i>lys⁺Sp⁺trp⁻</i>	48	
P20, <i>trpC2</i>	<i>lys-I, (hisB2)</i>	<i>lys⁺P20trp⁺</i>	12	<i>lys, P20, trp</i>
		<i>lys⁺P20trp⁻</i>	52	
		<i>lys⁺Sp⁺trp⁺</i>	61	
		<i>lys⁺Sp⁺trp⁻</i>	0	

Table 3. *Percentage recombination determined by two-factor transduction crosses of five Osp and four Sp⁻ mutants all reaching stage IV of spore development*

Heat resistance values were obtained at 7 h after transfer of cells into a medium to initiate sporulation and are taken from a previous paper (Coote, 1972). They were expressed as a percentage of the wild-type values which were taken as 100.

Mutant	Type	Heat resistance	Percentage recombination with auxotrophic markers		
			<i>leu-8</i>	<i>phe-12</i>	<i>lys-1</i>
x8	Osp	1.0	39	3	100
Z33A	Osp	1.0	51	11	100
Z5	Osp	0.2	43	6	100
Z24	Osp	0.5	100	76	99
Z28	Osp	0.1	100	85	97
E31	Sp ⁻	< 0.0001	100	75	96
Z7	Sp ⁻	< 0.0001	100	76	98
X2	Sp ⁻	< 0.0001	100	88	96
W3	Sp ⁻	< 0.0001	100	91	97

showed no linkage with the available markers. These genes presumably lie in areas of the map not covered by the markers used here or are due to multiple unlinked lesions.

Transformation analysis of stage IV mutants linked to the phe locus. Linked to the *phe-12* marker are five Osp mutations (Fig. 3) which cause the bulk of the cells to be blocked at stage IV in the sporulation process. They are Osp to varying degrees (Table 3), but the majority of cells in each case produced feebly refractile prespores under sporulation conditions (Coote, 1972). Eventually the mother cells lysed and released the unfinished spores into the medium. The feeble refractility and the degeneration of the mother cell were plainly visible in the phase-contrast microscope and facilitated a clear recognition of this phenotype. Four Sp⁻ mutants with an identical phenotype were also shown to be linked to the *phe-12* marker (Table 3). One of these (E31) was isolated by S. C. Warren and has been previously described (Piggot & Mandelstam, 1972).

The purpose of the analysis by transformation was to try to determine whether an Osp mutation and a Sp⁻ mutation were located within the same gene. Unfortunately, no Sp⁻ stage IV mutations were closely linked to the *phe-12* marker (Table 3) so the group loosely linked to this marker and which included both Sp⁻ and Osp mutations had to be studied. It was assumed that the mutations loosely linked by transduction to the *phe-12* marker would not be linked to this marker by transformation in which a much smaller piece of DNA

Table 4. *Reciprocal crosses by transformation of stage IV mutants linked by transduction to the phe-12 marker*

Values are R.I. $\times 100$. NR means not recorded as the numbers of *trp*⁺ transformants from these crosses were too small to be meaningful. In all other instances between 250 and 1500 *trp*⁺ transformants were scored for Sp⁺ or mutant phenotype.

Recipient (<i>trp</i> ⁻)	Donor DNA (<i>trp</i> ⁺)				
	Z7 Sp ⁻	X2 Sp ⁻	Z28 Osp	E31 Sp ⁻	W3 Sp ⁻
Z7	0.0	14.8	1.8	23.6	2.5
X2	11.3	0.0	8.7	5.3	15.4
Z28	3.2	7.3	0.0	3.0	3.6
E31	20.8	10.5	6.2	0.0	13.0
W3	NR	9.0	NR	NR	0.0
W5	87.0	92.0	110.0	88.0	121.0

Table 5. *Examples of the ratios of Sp⁺ to trp⁺ transformants obtained by reciprocal transformation*

The figures show the number of Sp⁺ recombinants (numerator) and the total number of *trp*⁺ transformants (denominator). The ratios used in calculating the recombination index (see Methods) are shown in parentheses.

Recipient (<i>trp</i> ⁻) ...		Donor DNA (<i>trp</i> ⁺)			
		Z7 Sp ⁻	Z28 Osp	E31 Sp ⁻	Wild-type —
Z7	Expt 1	0/1270	1/941 (0.0011)	14/1094 (0.013)	51/850 (0.06)
	Expt 2	0/944	0/646	7/395 (0.018)	66/926 (0.07)
Z28	Expt 1	2/840 (0.0024)	0/235	2/503 (0.004)	23/219 (0.11)
	Expt 2	3/818 (0.0036)	0/472	2/1108 (0.0018)	33/412 (0.08)
E31	Expt 1	9/513 (0.018)	2/712 (0.0028)	0/255	95/935 (0.1)
	Expt 2	3/310 (0.0097)	2/515 (0.0039)	0/889	16/395 (0.04)

is transferred than in PBS-1 transduction (Dubnau, Goldthwaite, Smith & Marmur, 1967). The unavailability of a linked marker for these mutations prompted the use of the recombination index method (see Methods). This procedure avoids variations in competence of recipients and variations in efficiency of DNA preparations by relating the transfer of a given marker to the transfer of an outside unlinked marker, in this case tryptophan dependence.

Reciprocal transformation was performed by the procedure described in Methods. The results (Table 4) suggest that all five mutations loosely linked to *phe-12* are closely linked to each other. The ratio of Sp⁺/*trp*⁺ recombinants used in calculating the recombination index was in all cases the average from at least two separate transformations. Examples of actual numbers obtained for three linked mutations are given in Table 5. Mutant Z24 yielded only an average of 0.2% (instead of the expected 5 to 10%) Sp⁺ colonies among the total *trp*⁺ transformants when used as recipient with wild-type DNA as donor and no increase in the number of Sp⁺ colonies was obtained if the DNA concentration was increased to 5 µg/ml. Z24 was presumed therefore to be a double mutant, was not further investigated and is not included in Table 4. Mutant W5 was included as a control and it showed no linkage to any other mutation which was in agreement with its location in the *metA*, *ura-1* region of the chromosome. No Sp⁺ colonies were detected when DNA prepared from the *trp*⁺

derivative of a mutant was used to transform the same *trpC* strain to prototrophy. This indicated that back-mutation of the recipients was not an important factor, so that although the numbers of Sp^+ colonies counted in some crosses were extremely low (Table 5), they were nevertheless considered to be significant.

DISCUSSION

Only one of the Osp mutations mapped p9, lying between *cysB* and *hisA*, has been located in one of the regions of the genome where tricarboxylic acid cycle mutations have been shown to map (Rutberg & Hoch, 1970). This indicates that the selection procedure used during isolation of the mutants (Coote, 1972) was probably successful in excluding mutants which were oligosporogenous because of a damaged tricarboxylic acid cycle.

The majority of the Osp mutations were located between the *phe-12* and *lys-1* markers near the terminal end of the chromosome where a majority of Sp^- mutations have been previously shown to map (Hoch & Spizizen, 1969; Rogolsky, 1969; Takahashi, 1969; Ionesco *et al.* 1970; Piggot & Mandelstam, 1972). All but two of the remainder of the Osp mutations showing linkage were situated in the region which stretched from *cysB* to the right of *ura-1* in an area also occupied by Sp^- genes (Rogolsky, 1969; Ionesco *et al.* 1970; Piggot & Mandelstam, 1972).

It is worth noting that the positions of some of the Osp mutations within the *phe-12*, *lys-1* segment are very similar to those of previously located Sp^- mutations. Stage II Sp^- mutations have been placed a similar distance from *lys-1* as p18 (Ionesco *et al.* 1970; P. Piggot, personal communication). The stage O and I Osp mutations lie between 30 and 50 units to the left of *lys-1* and similar Sp^- mutations have been located between 35 and 50 units to the left of this marker (Hoch & Spizizen, 1969; Hoch, 1971). In addition stage III and IV Sp^- mutations have been reported in similar positions in this region to the stage III and IV Osp mutations (Hoch & Spizizen, 1969; Ionesco *et al.* 1970; Piggot & Mandelstam, 1972). In so far as it is possible to compare transduction data from different laboratories these similar groupings of the Osp and Sp^- mutations are consistent with the idea that mutation in one gene can produce either an Osp or a Sp^- phenotype. The stage IV Osp mutations loosely linked to *phe-12* also showed a weak linkage to *lys-1* which is in agreement with the genetic continuity recently found in this region (Ionesco & Cami, 1969; Ionesco *et al.* 1970). Only one Osp mutation was located to the right of the *lys-1* marker and this produced phenotypic effects not previously described in sporulation of *Bacillus subtilis* (Coote, 1972). It was characterized by an accumulation of spore coat material in the mother cell cytoplasm instead of around the developing spore. It is possible that a gene concerned with the control of coat deposition is situated to the right of *lys-1*. A Sp^- mutation producing a very similar phenotype has also been located between the *lys-1* and *trpC* markers (P. Piggot, personal communication).

The results of the mapping bring out other points that Osp and Sp^- mutants have in common. Thus, Osp mutations associated with the same type of morphological block can be widely separated on the chromosome in the same way as Sp^- mutations (see Ionesco *et al.* 1970; Piggot & Mandelstam, 1972). For example, mutants p14 and p18, both of which have a similar sporulation phenotype and which lay down septa at both ends of the cell, map at opposite ends of the genome. This presumably means that interference with at least two distinct biochemical steps can produce this particular aberration. Conversely genes situated very close to each other can apparently be associated with totally different structural events. In the chromosomal segment between the *phe-12* and *lys-1* markers stage O, II, III and IV phenotypes are present in close juxtaposition (Fig. 3).

The possibility that mutations of similar morphology were located within a single gene cannot be judged from recombination data using transduction which are too variable to establish this (see also Hoch, 1971). For instance, in separate transduction experiments the percentage co-transfer with the *phe-12* marker of mutation z28 was 13, 10, 18 and 19% and for mutation z7 the values with this marker were 26, 20, 21 and 28%. For analysis of mutations which are closely linked transformation is more suitable because recombination between closely linked mutations is more likely to occur when the number of genes transferred in the process is so much smaller. The transformation data (Table 4) show that the mutations loosely linked to *phe-12* by transduction (z28, E31, z7, x2 and w3) were all closely linked to each other. The number of cistrons comprising this linkage group could not be directly determined because without diploidy in *Bacillus subtilis* direct complementation is not possible. However, the maximum R.I. can be compared with that obtained from the most distant markers of mutations known to be within a single gene. In experiments with *B. subtilis* 168 Carlton (1966) found a R.I. $\times 100$ of 20 for the most distant markers within the tryptophan synthetase gene and values as high as 30 were recorded during the analysis of ornithine transcarbamylase mutants (Mahler, Neumann & Marmur, 1963). It seems reasonable to take a value of 10 or less as probably representing one gene and this has been done previously by Rouyard *et al.* (1967). Recombination indices (Table 4) of less than 10 were obtained between the Osp mutation z28 and both Sp⁻ mutations z7 (1.8 and 3.2) and E31 (3.0 and 6.2). The reciprocal crosses shown in Table 4 gave for the most part values which were in good agreement, but not accurate enough to ascribe an unequivocal order to the mutations. The method used suffers from the weakness for the need to count a large number of transformants, and without direct selection for Sp⁺ recombinants only a very small number of these are detected in a cross between two closely linked mutations.

The work of Rouyard *et al.* (1967) showed that reciprocal transformation between a Sp⁻ and an Osp stage II mutant yielded R.I. $\times 100$ values of between 0 and 3.0. This seems to be the only other direct evidence presented to indicate that mutations in one gene may produce either the Sp⁻ or the Osp phenotype. Low recombination values would also be obtained if the mutations were located very close to each other at the ends of adjacent genes. They would then be functionally separate, but recombination will take place across the boundary of the two genes. The recombination data (Table 4) place Osp mutation z28 between the Sp⁻ mutations z7 and E31 as z7 and E31 have reciprocal values of 20.8 and 23.6. It seems most unlikely from the recombination values that these mutations span the length of three genes unless z28 is a mutation in a very small regulatory area lying between two larger genes. If this order of the mutations is correct then even if z7 and z28 were at the extreme ends of separate loci, it seems likely that E31 (Sp⁻) at least has a mutation in the same gene as z28 (Osp). Another possible complicating factor is that mutants z7 and z28 were derived by treatment of the wild-type with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a mutagen which causes induction of closely linked multiple mutations in *Escherichia coli* (Guerola, Ingraham & Cerdá-Olmedo, 1971). However, reciprocal crosses (Table 4) gave similar recombination values which makes the possibility of two linked spore mutations in one or both mutants of a cross unlikely as a reason for the low numbers of Sp⁺ recombinants.

The possibility cannot be eliminated that some Osp mutations are of a distinct regulatory nature. However, the fact that many Osp mutants have phenotypically similar Sp⁻ counterparts (Coote, 1972), the marked similarity in the location on the genome of the two types of mutation and the evidence presented here and elsewhere (Rouyard *et al.* 1967) suggesting the location of an Osp mutation and its Sp⁻ counterpart within a single gene indicate strongly

that some Osp strains at least are probably leaky mutants. Thus, although the distinction between the two types of mutant is phenotypically very clear it may not necessarily involve separate genes for the Sp⁻ and Osp phenotypes.

I am very grateful to Dr P. Piggot and Professor J. Mandelstam for many helpful discussions. I thank Mrs Dawn Torgerson for skilled technical assistance. The work was supported by a grant from the Science Research Council.

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