New Types of Mutation Affecting Formation of Alkaline Phosphatase by *Bacillus subtilis* in Sporulation Conditions

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Mutations defining three new loci, *sapA*, *sapB* and *phoS*, were detected by their ability to overcome the phosphatase-negative phenotype of early-blocked asporogenous mutants in sporulation conditions. Synthesis of alkaline phosphatase by *Bacillus subtilis* is subject to 'vegetative' and 'sporulation' controls. The *phoS* mutations resulted in constitutive production of alkaline phosphatase and so could be altered in either the 'vegetative' or the 'sporulation' control system. The *sapA* and *sapB* mutations only affected alkaline phosphatase formation in sporulation conditions, and were considered to be sporulation specific. They rendered 'sporulation' alkaline phosphatase formation independent of all the *spo* mutations tested, and so independent of the control of the dependent sequence of *spo* locus expression; as the enzyme was not formed constitutively, it remained subject to some other sporulation control.

The *sapA* and *phoS* loci were placed between *argC4* and *metC3* on the genetic map; the *sapB* locus was located close to *purB6*. The three loci mapped separately from all known *spo* loci.

INTRODUCTION

Bacterial spore formation is a form of cell development. As such it serves as a model for cell development in other, more refractory systems. For *Bacillus subtilis* more than 30 *spo* loci have been identified by mutations that prevent sporulation but do not affect vegetative growth (Hoch, 1976; Piggot & Coote, 1976). As the loci are not expressed during exponential growth it seems likely that they are 'switched on' during spore formation. As most of the loci are well separated on the chromosome, they must have their own switch-on mechanism. For this reason the loci can be considered as sporulation operons (Piggot, 1973; Schaeffer *et al.*, 1965). Statistical considerations suggest that 40 to 50 such operons may be specifically expressed during sporulation (Hranueli, Piggot & Mandelstam, 1974).

The pleiotropic effects of different *spo* mutations fit into a pattern suggesting that spore formation proceeds by a dependent sequence of events (Balassa, 1969; Waites *et al.*, 1970). In this, the occurrence of any sporulation event is dependent on the correct occurrence of all previous events. This can be represented schematically as $A \rightarrow B \rightarrow C \rightarrow D \dots$, where D is dependent on C etc. The detailed way in which the expression of 40 to 50 sporulation operons is organized in such a dependent sequence (or dependent sequences) is not known. However, some features are already apparent. Thus the scheme must include sequential transcription of sporulation operons as stage-specific mRNA have been demonstrated (DiCioccio & Strauss, 1973; Sumida-Yasumoto & Doi, 1974). Control of spore coat biosynthesis did not fit neatly into a dependent sequence (Aronson & Fitz-James, 1976; Wood,

1972). However, as spore coat constituted about 50 % of total spore protein (Aronson & Fitz-James, 1976), it may reasonably be regarded as a special case. The epistatic relationships of a series of *spo* mutations were consistent with a single linear sequence of sporulation operon expression at least till stage III (prespore free within the mother cell) of sporulation (Coote & Mandelstam, 1973). The results reported for mutations causing blocks after stage III could not easily be reconciled with a single sequence, although they would be compatible with two sequences, one within the developing spore and the other within the mother cell.

A reasonable approach to an understanding of the way in which the network of gene expression is organized would seem to be to isolate mutants that are altered in the control of the dependent sequence of events. In principle, to do this, an early blocked asporogenous mutant that is negative for all the later sporulation events can be treated with mutagen, and secondary mutants isolated in which some (at least) of these later events are expressed. If the original *spo* mutation is retained, then the second mutation has partly overcome the pleiotropy of the original and may be regarded as a putative control mutation. We have used this approach, and chosen as our 'late' event the formation of alkaline phosphatase (EC 3.1.3.1) which is associated with stage II (asymmetric septation) of sporulation (Waites *et al.*, 1970).

Alkaline phosphatase synthesis is repressed by inorganic phosphate during exponential growth (Anagnostopoulos, 1960), but it is formed in the presence of inorganic phosphate during sporulation (Glenn & Mandelstam, 1971; Warren, 1968). It is convenient to distinguish these two activities as 'vegetative' and 'sporulation' alkaline phosphatase respectively. Two closely-linked loci, *phoP* and *phoR*, have been shown to control the formation of the enzyme during exponential growth (Le Hégarat & Anagnostopoulos, 1969; Miki, Minima & Ikeda, 1965; Nukushina & Ikeda, 1969). Mutation in these loci can give a completely repressed, *phoP*, or a constitutive, *phoR*, phenotype for the vegetative enzyme. These mutations did not affect sporulation, and the *phoP* mutations did not prevent formation of the enzyme during sporulation (Grant, 1974; Ichikawa & Freese, 1974). In contrast, mutations in any one of several *spo* loci prevented formation of the sporulation enzyme (Piggot & Coote, 1976; Waites *et al.*, 1970), but did not affect formation of the vegetative enzyme (Glenn & Mandelstam, 1971; P. J. Piggot, unpublished observations). Glenn & Mandelstam (1971) have provided strong evidence that the enzyme which appears during sporulation is the same protein as the enzyme synthesized by vegetative bacteria upon phosphate depletion. Consequently the distinction between vegetative and sporulation alkaline phosphatase is between control systems rather than between actual enzymes.

Sporulation alkaline phosphatase was not formed by strains with *spoIIA*, *spoIIE*, *spoIIF* or *spoIIG* mutations, nor by any of the strains tested that have *spoO* mutations. However, the enzyme was formed by strains with mutations in the *spoIIB*, *spoIIC* or *spoIID* loci or in any locus associated with a later stage (Piggot & Coote, 1976). Thus enzyme formation was closely associated with the dependent sequence of sporulation events. We report here the isolation of mutants in which the sporulation phosphatase-negative phenotype of early-blocked *spo* mutants is reversed. The existence of separate vegetative and sporulation control systems complicates interpretation of results for the mutants that produce the enzyme constitutively. However, the secondary mutations that only affect formation of the enzyme under sporulation conditions are thought to be control mutations disrupting the sequence of sporulation events leading to alkaline phosphatase formation. A preliminary account of this work was presented at the British Spores Group Meeting (Leeds, December 1975).

METHODS

Strains. *Bacillus subtilis* 168 (*trpC2*) was used as the standard strain and is referred to as the wild type. Other strains used were derivatives of this, and are listed in Table 1. The nomenclature of *spo* loci conforms to the system described previously (Piggot & Coote, 1976). The convention introduced by Ryter (1965) is used in numbering the cytological stages of sporulation.

Table 1. *Strains of B. subtilis used*

Strain	Genotype	Origin and/or reference
Auxotrophic, sporulating strains		
BD70	<i>metC3</i>	D. Dubnau
BD163	<i>hisA1 argC4</i>	D. Dubnau
GSY860	<i>argA11 nic-38</i>	C. Anagnostopoulos
GSY1020	<i>purB6 hisB2</i>	C. Anagnostopoulos
MB19	<i>leu-8 trpC2 rif-2</i>	Piggot (1973)
MB20	<i>leu-8 trpC2 rif-2 tal-1</i>	Spontaneous mutant of MB19, resistant to β -thienyl alanine
MB21	<i>leu-8 metC3 tal-1</i>	DNA from BD70 used to transform MB20
MB60	<i>leu-8 purB6 rif-2</i>	DNA from QB693 used to transform MB19
MB61	<i>trpC2 purB6 rif-2</i>	DNA from GSY1020 used to transform MB19
QB693	<i>purB6 tre-12</i>	J.-A. Lepesant
60935	<i>dal metC trpC</i>	E. Freese
Strains with <i>spo</i> mutations		
JH137	<i>trpF7 spoOA12 sus-3</i>	J. A. Hoch
17.1	<i>spoOH17 rif-2 trpC2</i>	Previously designated E22 (Piggot, 1973)
17.8	<i>spoOH17 nic-38 argA11 rif-2</i>	GSY860
17.10	<i>spoOH17 argC4 hisA1 rif-2</i>	BD163
17.11	<i>spoOH17 purB6 hisB2 rif-2</i>	GSY1020
17.13	<i>spoOH17 metC3 leu-8 tal-1 rif-2</i>	MB21
17.21	<i>spoOH17 tre-12 rif-2</i>	QB693
17.22	<i>spoOH17 dal metC trpC rif-2</i>	60935
34.1	<i>spoOA34 phe-12 rif-2 tal-1</i>	Hranueli <i>et al.</i> (1974)
55.1	<i>spoIIIG55 leu-8 tal-1</i>	Previously designated NG12.12 (Piggot, 1973)
55.2	<i>spoIIIG55 trpC2 rif-2</i>	Previously designated NG15.4 (Piggot, 1973)
61.1	<i>spoIIIE61 trpC2 rif-2</i>	
64.1	<i>spoIIIE64 leu-8 rif-2</i>	Previously designated NG17.15 (Piggot, 1973)
69.1	<i>spoIIA69 trpC2 rif-2</i>	Previously designated NG18.6 (Piggot, 1973)
69.3	<i>spoIIA69 purB6 rif-2</i>	MB61
96.2	<i>spoIIF96 metC3 tal-1</i>	Hranueli <i>et al.</i> (1974)
96.3	<i>spoIIF96 cysC7</i>	M. Young
261	<i>spoIIA69 spoIIIG55 rif-2</i>	<i>spoIIA69</i> transferred into 55.2 by cotransduction with <i>trp</i> ⁺
262	<i>spoIIA69 spoIIIE61 rif-2</i>	<i>spoIIA69</i> transferred into 61.1 by cotransduction with <i>trp</i> ⁺
Phosphatase mutants		
300.1	<i>sapB2 spoIIA69 rif-2</i>	
300.3	<i>sapB2 spoIIF96 rif-2</i>	
300.5	<i>sapB2 spoIIIG55 rif-2</i>	
300.6	<i>sapB2 spoIIIE64 rif-2</i>	
300.12	<i>sapB2 spoOA12 rif-2</i>	
300.13	<i>sapB2 leu-8 rif-2</i>	
300.14	<i>sapB2 trpC2 rif-2</i>	
300.16	<i>sapB2 spoIIIG55 leu-8 rif-2</i>	
302.1	<i>phoR2 spoIIA69 rif-2</i>	
304.1	<i>sapA4 spoIIA69 rif-2</i>	
304.7	<i>sapA4 metC3 tal-1</i>	
305.1	<i>phoS5 spoIIA69 rif-2</i>	
305.7	<i>phoS5 leu-8 tal-1</i>	
306.1	<i>sapA6 spoIIA69 rif-2</i>	
306.3	<i>sapA6 spoIIF96 tal-1</i>	
306.5	<i>sapA6 spoIIIG55 tal-1</i>	
306.7	<i>sapA6 leu-8 tal-1</i>	
306.15	<i>sapA6 spoOA34 tal-1</i>	
308.1	<i>phoS8 spoIIA69 rif-2</i>	
308.7	<i>phoS8 leu-8 tal-1</i>	
308.9	<i>phoS8 leu-8 spoIIIE64 rif-2 tal-1</i>	
309.1	<i>phoR9 spoIIA69 rif-2</i>	
310.1	<i>sapB10 spoIIA69 rif-2</i>	
310.14	<i>sapB10 trpC2 rif-2</i>	

Table 2. *Confirmation of genetic constitution of constructed double mutants containing sapB2*

Linkage values are given as percentage cotransduction. The numbers of transductant colonies counted are indicated in parentheses (no. of selected transductants with unselected donor marker/total no. of selected transductants). The anticipated linkage value for *spoOA12* is that given by Hoch & Nester (1973), and for *spoIIA69*, *spoIIE64*, *spoIIG55* by Piggot (1973).

Strain	Relevant genotype	Strain construction	Linkage of <i>sap</i> to <i>purB6</i>	Linkage of <i>spo</i>		
				Marker	Anticipated	Found
300.1	<i>sapB2 spoIIA69</i>	69.1 transformed by DNA from original mutant isolate	67 (72/107)	<i>lys-1</i>	94	91 (21/23)
300.3	<i>sapB2 spoIIF96</i>	300.13 transformed by DNA from 96.3	85 (58/68)	No Spo ⁺ recombinants with 96.2 as recipient		
300.5	<i>sapB2 spoIIG55</i>	300.14 transformed by DNA from 55.1	88 (277/314)	<i>ura-1</i>	83	70 (35/50)
300.6	<i>sapB2 spoIIE64</i>	64.1 transformed by DNA from original mutant isolate	82 (474/581)	<i>cysA14</i>	93	75 (135/179)
300.12	<i>sapB2 spoOA12</i>	300.13 transformed by DNA from JH137	74 (660/890)	<i>aroD120</i>	21	12 (12/99)
300.13	<i>sapB2 leu-8</i>	MB60 transduced to <i>pur</i> ⁺ with lysate prepared on 300.6	71 (53/75)	—	—	—
300.14	<i>sapB2 trpC2</i>	MB61 transduced to <i>pur</i> ⁺ with lysate prepared on 300.6	77 (30/39)	—	—	—
300.16	<i>sapB2 spoIIG55</i>	55.1 transformed by DNA from 300.1	74 (320/432)	<i>ura-1</i>	83	25 (188/744)

Media. Most of the media used have been described previously (Piggot, 1973). High phosphate medium was the lactate-glutamate-minimal medium adjusted to 3.0 mM-phosphate with sodium phosphate (pH 7.0). The medium used to select trehalose-utilizing recombinants was that described by Lepesant-Kejzlarova *et al.* (1975). Samples were diluted in Spizizen's minimal medium (1958) supplemented with 0.2 % (w/v) glucose. Basal medium has been described previously (Piggot, 1975). Minimal growth medium was the sporulation medium of Sterlini & Mandelstam (1969) supplemented with glucose (0.2 %, w/v).

Isolation of 'sporulation' alkaline phosphatase mutants. Exponentially growing cultures of strains 261 and 262 were treated with ethyl methanesulphonate as described by Coote (1972). The cultures were then grown overnight at 35 °C in Penassay Broth (Difco). These were diluted to 1 in 10⁵ and 0.2 ml was plated on nutrient agar (Difco). Plates were incubated for 24 h at 42 °C. They were then stained for alkaline phosphatase using a fresh solution of fast blue RR salt (Sigma) and α -naphthyl acid phosphate (each at 1 mg ml⁻¹) in 1.0 M-Tris/HCl, pH 8.0. 'Vegetative' alkaline phosphatase was repressed under these conditions. Phosphatase-positive colonies were picked and isolated by restreaking on nutrient agar. It was necessary to pick mutant colonies as soon as they were distinguishable from those of the parent strain in order to circumvent the toxic effects of the stain. Each of the mutants described in this paper resulted from a separate mutagenic treatment. The mutants were checked for a nonsense-type suppressor by their ability to support plaque formation of bacteriophages SPO-1 and SPO-1*sus*-5. It was concluded that none of the mutants contained such a suppressor; this was supported by the observation that none of the strains had regained the ability to sporulate. DNA was prepared from each of the mutants, and the mutation was transferred into strain 69.1 by transformation with saturating concentrations of DNA (congression; Nester, Schafer & Lederberg, 1963) selecting for Trp⁺ recombinants. The resulting strains 300.1, 302.1 etc. (Table 1) were used in subsequent studies unless otherwise indicated. For the eight mutants described here, the mutations have behaved as single mutations in all genetic crosses. The lesions in the mutants which formed alkaline phosphatase during vegetative growth in high phosphate media as well as in sporulation conditions are designated *pho*. The lesions that resulted in enzyme formation in sporulation conditions but not in vegetative growth are designated *sap* (sporulation alkaline phosphatase). A number of derivatives of each of the *pho* and *sap*

mutants were constructed (Table 1). Each constructed strain was then checked for the presence of the appropriate markers. This is illustrated for the set derived from mutant 300 (Table 2).

Mapping by transduction of mutations affecting 'sporulation' phosphatase formation. As Spo^+ strains form sporulation alkaline phosphatase, it was assumed that *sap* recombinants might only be readily detectable in Spo^- strains that were ordinarily negative for the sporulation phosphatase. Consequently the *spoOH17* mutation was transferred into a series of auxotrophs by cotransformation with *rif* (Table 1). The phosphatase mutations were then mapped by bacteriophage PBS1-mediated transduction using these asporogenous auxotrophs as recipients. The procedure for transduction described previously (Piggot, 1973) was modified slightly for asporogenous recipients in that it was found more satisfactory to grow these in basal medium plus growth requirements. When possible, transductants were selected on high phosphate minimal agar and transductant colonies were stained directly for alkaline phosphatase. Prototrophic transductants of all *purB6* strains tested grew very poorly on this medium. The reason for this was not discovered, but the transductants retained this property even after isolation on nutrient agar, so that it was not a problem of the initial selection. The difficulty was overcome by supplementing minimal agar with 0.01 % (w/v) glucose. When other media were used for the primary selection, transductant colonies were picked, isolated on nutrient agar, and then stained for alkaline phosphatase.

Transformation. The method used for transformation was that of Piggot (1973).

Transformation crosses between phosphatase mutants. The transduction crosses suggested that several of the *sap* and *pho* mutations mapped close together. To determine the extent to which this was true, mutants were crossed against each other by transformation and the distance between mutations was measured as the recombination index (Lacks & Hotchkiss, 1960). An unlinked *spo* mutation served as internal standard. For example, if we consider donors *sap-1 spo⁺* and *sap⁺ spo⁺* and recipient *sap-2 spo⁺*, then the recombination index (RI) between *sap-1* and *sap-2* is given by the ratio of transformants [*sap⁺ spo⁺* (*sap-1* as donor)]/[*sap⁺ spo⁺* (*sap⁺* as donor)]. The RI is 1.0 when the *sap* mutations are unlinked, and decreases the more closely they are linked.

In practice the *sap* (or *pho*) markers were not selective. Consequently saturating concentrations of DNA were used, and primary selection was for transformation of the recipient, which was also auxotrophic, to prototrophy. Under these conditions up to 5 % of the prototrophic transformants would be expected to receive an unlinked mutation (such as *sap* or *spo*) from the donor as a result of a second transformation event (Nester *et al.*, 1963). The ratio of *sap⁺* (or *pho⁺*)/*spo⁺* colonies amongst the prototrophic transformants was then determined. The number of *spo⁺* colonies was obtained by a direct count of pigmented colonies (Iichinska, 1960; Schaeffer & Ionesco, 1960). The number of *sap⁺*, or *pho⁺*, colonies was obtained after staining the plates for alkaline phosphatase activity. Because of the nature of the cross (the recipient being *spo*), these were seen as a small number of phenotypically sporulation phosphatase-negative colonies amongst a large number of phosphatase-positive colonies. To determine the number unambiguously it was found necessary to incorporate the stain into a soft agar overlay (Miller, 1972). A minimum of 50 Spo^+ colonies were counted for each cross, and of 50 sporulation phosphatase-negative colonies in the control crosses.

Procedure for obtaining sporulation. The procedure used was the resuspension method described by Sterlini & Mandelstam (1969) except that L-alanine (1.58 g l⁻¹) was used in the growth medium rather than DL-alanine, and 2.25 ml growth medium was added to 1 l sporulation medium. All incubations were at 42 °C, and media were warmed to this temperature before use. Times were measured from the time of resuspension. With *B. subtilis* 168 phase-grey prespores began to appear after 3 h and by 4.5 h 80 to 90 % of the bacteria contained phase-grey to phase-bright bodies. Asporogenous mutants treated in this manner are referred to as being incubated in sporulation conditions.

Assay for alkaline phosphatase. Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as substrate in 0.5 M-diethanolamine/HCl, pH 10, at 30 °C, as described by Glenn & Mandelstam (1971). The spectrophotometer was calibrated with a standard solution of *p*-nitrophenol (Sigma). One unit of alkaline phosphatase activity is defined as the amount that catalyses the hydrolysis of 1 nmol *p*-nitrophenyl phosphate in 1 min. Specific activities are expressed as units (mg bacterial dry wt)⁻¹.

Assays for glucose dehydrogenase and for dipicolinic acid. Samples were assayed for glucose dehydrogenase (EC 1.1.1.47) by the method of Sadoff, Bach & Kools (1965), and for dipicolinic acid by the method of Janssen, Lund & Anderson (1958).

Growth. Growth of bacteria in culture was determined by measuring the $E_{600\text{ nm}}$ with a Unicam SP600 spectrophotometer. The $E_{600\text{ nm}}$ was converted to bacterial dry wt ml⁻¹ by means of a standard calibration curve.

Table 3. *Linkage by transduction of phosphatase mutations*

Linkage values are given as percentage cotransduction. The numbers of transductant colonies counted are indicated in parentheses [*sap*/(*sap* + *sap*⁺) or *pho*/(*pho* + *pho*⁺)].

Mutation	Linkage to selected marker	
<i>sapA4</i>	<i>metC3</i>	63 (248/397); <i>argC4</i> 22 (42/193)
<i>sapA6</i>	<i>metC3</i>	74 (139/189); <i>argC4</i> 23 (16/69)
<i>sapB2</i>	<i>purB6</i>	88 (277/314); <i>tre-12</i> 32 (9/28); <i>dal</i> 2 (12/536)
<i>sapB10</i>	<i>purB6</i>	74 (589/805); <i>tre-12</i> 26 (7/27); <i>dal</i> 3 (4/118)
<i>phoR2</i>	<i>argA11</i>	58 (235/409); <i>leu-8</i> 28 (55/199)
<i>phoR9</i>	<i>argA11</i>	61 (324/532); <i>leu-8</i> 43 (124/290)
<i>phoS5</i>	<i>metC3</i>	77 (143/185); <i>argC4</i> 37 (26/70)
<i>phoS8</i>	<i>metC3</i>	78 (392/500); <i>argC4</i> 27 (20/75)

RESULTS

From the original screening, eight phosphatase mutants were isolated and extensively characterized. Four were of the *sap* type, affecting the formation of alkaline phosphatase only in sporulation conditions, and four were of the *pho* type, affecting phosphatase formation during growth as well as in sporulation conditions.

Characterization of sap mutants

The location of the *sap* mutations on the *B. subtilis* chromosome was determined by bacteriophage PBS1-mediated transduction. The mutations mapped in two groups designated *sapA* and *sapB*. Mutations *sapA4* and *sapA6* were linked to *metC3* and *argC4* (Table 3). The cotransduction frequencies suggested that the mutations were close together. As the mutations resulted in similar phenotypes they were assumed to be in a single locus. Transformation crosses confirmed that *sapA4* and *sapA6* were close together (RI = 0.23). The *sapA4* mutation tended to revert, so that the RI is probably a considerable overestimate of the distance between the mutations. Because of the instability of *sapA4*, precise characterization was difficult, and most subsequent studies of *sapA* were with strains carrying *sapA6*. The *sapA* locus almost certainly lies between *metC3* and *argC4* since these mutations are only very weakly linked by PBS1-mediated transduction (Dubnau *et al.*, 1967).

The *sapB2* and *sapB10* mutations were closely linked to *purB6* by transduction (Table 3). The two mutations gave rise to indistinguishable phenotypes. The peak sporulation phosphatase activities of strains 300.1 and 310.1 were, respectively, 175 and 181 units (mg dry wt)⁻¹. No Sap⁺ transformants were obtained in transformation crosses between strains 300.16 and 310.1, so the mutations were assumed to lie in a single locus (from the control crosses, 50 Sap⁺ transformants would have been expected if *sapB2* and *sapB10* had been unlinked). The numbers were insufficient to decide whether the two mutations were in the same site. The mutations were also linked to *tre-12* (about 30 % cotransduction) and weakly linked to *dal* (Table 3). From the published linkage values for this region (Lepesant-Kejzlarova *et al.*, 1975) it is not possible to give a firm map position, although the most likely map order is *dal purB sapB tre*.

In growth medium the *sap* mutants had a very low basal level of enzyme with a specific activity of less than 3 units (mg dry wt)⁻¹, similar to that of the wild type. The specific activity only began to rise after resuspension in the sporulation medium, that of strain 300.1 (*sapB2 spoIIA69*) reaching 175 units (mg dry wt)⁻¹, and that of strain 306.1 (*sapA6 spoIIA69*), 15 units (mg dry wt)⁻¹ (Fig. 1). In contrast the specific activity of strain 69.1 (*sap*⁺ *spoIIA69*) did not rise significantly above the basal level. Glenn & Mandelstam (1971) noted that there was day-to-day variation in the specific activity of sporulation alkaline phosphatase produced by the wild type. Such a variation was particularly apparent for strain 306.1 where the maximum specific activity varied from 15 to 25 units (mg dry wt)⁻¹. A similar

Fig. 1

Fig. 2

Fig. 1. Specific alkaline phosphatase activity in sporulation conditions of the wild type (□), strain 69.1 (●, *spoIIA69*), and of *spoIIA69* strains harbouring different phosphatase mutations: ○, 300.1 (*sapB2 spoIIA69*); △, 306.1 (*sapA6 spoIIA69*); ■, 308.1 (*phoS8 spoIIA69*).

Fig. 2. Production of alkaline phosphatase by different *spo*⁺ strains growing in minimal growth medium: ○, □, 305.7 (*phoS5*, different experiments); △, ▽, 308.7 (*phoS8*, different experiments); ▼, 300.13 (*sapB2*); ▲, 304.7 (*sapA4*); ■, 306.7 (*sapA6*); ●, wild type. Enzyme units and bacterial dry wt values are for culture samples of 1 ml.

absolute variation was also found for 300.1, but proportionately was much less significant. Alkaline phosphatase was not formed when chloramphenicol (50 µg ml⁻¹) was added at the time of resuspension.

The sporulation system involved transfer from a rich medium containing casein hydrolysate (1.0 %, w/v) to a poor medium containing glutamate (0.2 %, w/v) as sole carbon source (Sterlini & Mandelstam, 1969). Consequently, it was possible that phosphatase synthesis was the result of the poor medium *per se* rather than of the sporulation process. To test this, bacteria were induced to grow exponentially in the sporulation medium supplemented with 0.2 % (w/v) glucose (minimal growth medium). Under these conditions none of the *sap* mutants formed enzyme to an extent that was significantly different from the basal level produced by the wild type (Fig. 2). It seems reasonable to conclude that alkaline phosphatase formation by *sap* mutants was a consequence of the sporulation process rather than of the poor medium.

The *sapA* and *sapB* mutations did not overcome the pleiotropic effects of the *spoIIA69* mutation on the other sporulation parameters tested. Strains 300.1 and 306.1 did not produce detectable levels (< 5 % of wild type) of either glucose dehydrogenase or dipicolinic acid, so the *sap* mutations did not restore the ability to synthesize these compounds. The *spoIIA69 sap* mutants retained the abortively disporic asporogenous phenotype (as seen by phase contrast microscopy) that characterized the *spoIIA69 sap*⁺ strain, 69.1 (NG18.6 of Piggot, 1973).

Expression of *sap* mutations in different backgrounds

Neither the *sapA* nor the *sapB* mutations interfered with spore formation in strains that were *spo*⁺. With *sapB*, the *spo*⁺ strain showed the pattern of alkaline phosphatase synthesis associated with *sapB* rather than *sap*⁺ (compare the wild type and 300.1, Fig. 1, with 300.13

Fig. 3. Specific alkaline phosphatase activity in sporulation conditions of different strains carrying *sapB2*: ●, 300.1 (*sapB2 spoIIA69*); □, 300.3 (*sapB2 spoIIF96*); △, 300.5 (*sapB2 spoIIG55*); ○, 300.6 (*sapB2 spoIIE64*); ▲, 300.12 (*sapB2 spoOA12*); ■, 300.13 (*sapB2 spo⁺*).

and 300.1, Fig. 3). The difference between *sapA6 spoIIA69* and *sap⁺ spo⁺* (Fig. 1) was not sufficiently clear cut to decide which pattern the *sapA6 spo⁺* strain showed.

The *sapA* and *sapB* mutations were expressed in all the early-blocked *spo* mutants tested. These included strains with *spoOA* mutations, the most pleiotropically negative of all *spo* mutations (Brehm, Staal & Hoch, 1973; Michel & Cami, 1969; Piggot & Coote, 1976), and also with *spoIIA*, *spoIIE*, *spoIIF* and *spoIIG* mutations. None of the *spo sap⁺* mutants produced a significant level [specific activity < 2.5 units (mg dry wt)⁻¹] of sporulation alkaline phosphatase.

The day-to-day variation in the amount of sporulation alkaline phosphatase did not significantly affect the large amounts of enzyme produced by *sapB* mutants, and there were consistent quantitative variations between *sapB* strains with different *spo* mutations (Fig. 3). The *spo* mutations fitted the pattern *spoOA* [peak specific activity 250 units (mg dry wt)⁻¹] > *spoIIA*, *spoIIE* [175 to 150] > *spoIIF* [110], *spoIIG* [80]. As *spoOA* mutations result in reduced protease activity (Brehm *et al.*, 1973; Michel & Cami, 1969) and alkaline phosphatase is susceptible to proteolysis (Glenn & Mandelstam, 1971), these differences could be, at least partly, a consequence of different proteolytic activities.

The day-to-day variation in enzyme production by *sapA* mutants effectively disguised any differences that may have existed between these mutants. Nevertheless, there was no indication that the *sapA spoOA* strain formed significantly more enzyme than the other *sapA* strains. Thus variation in proteolysis is unlikely to be a sufficient explanation for the differences that were seen with *sapB* strains or for the day-to-day variations in alkaline phosphatase activity.

Characterization of *pho* mutants

The mutations from the four *pho* mutants mapped in two distinct sites. Two of the mutations (from strains 302.1 and 309.1) mapped in the region of the chromosome, near *argA* (Table 3), where the *phoR* locus has already been located (Miki *et al.*, 1965; Nukushina & Ikeda, 1969). The strains produced enzyme constitutively, but were not fully derepressed, and were similar to certain *phoR* mutants (Miki *et al.*, 1965). The mutations were presumed to lie in the *phoR* locus and are not considered further.

The mutations from strains 305.1 and 308.1 mapped between *metC3* and *argC4* by transduction (Table 3). No *pho* mutations have previously been shown to map in this region, and so they identify a new locus, designated *phoS*. The two mutations were closely linked to each other ($RI = 0.04$) and also to the two *sapA* mutations ($RI = 0.06$ to 0.40) in transformation crosses. From the mapping data, the four mutations could lie in a single locus. However, there was a clear phenotypic distinction between strains carrying the *phoS* (305.7 and 308.7) and the *sapA* (304.7 and 306.7) mutations (Fig. 2). Bacteria were induced to grow exponentially in the minimal growth medium. Under these conditions the *phoS* mutants produced alkaline phosphatase constitutively whereas the *sapA* mutants only produced the basal level characteristic of the wild type. It is thought that the most reasonable interpretation of the data is two adjacent loci, *sapA* and *phoS*.

There was a consistent difference between strains with the *phoS5* and *phoS8* mutations in the specific alkaline phosphatase activity during exponential growth [strains 305.7 and 308.7 had specific enzyme activities of 170 and 60 units (mg dry wt)⁻¹ respectively, Fig. 2]. Similar quantitative differences have also been reported for different *phoR* mutants (Miki *et al.*, 1965). The reason for the fall in specific enzyme activities at higher bacterial densities (Fig. 2) is not known. At these densities growth was no longer exponential, and the relative loss of alkaline phosphatase activity could have resulted from increased proteolytic activity as the growth rate declined.

The constitutive levels of *phoS* mutants did not approach the fully derepressed level. Bacteria (strain 305.1 and 308.1) growing exponentially in the minimal growth medium were harvested by centrifugation and resuspended in minimal growth medium lacking phosphate. Within 1 h the specific alkaline phosphatase activity was about 1000 units (mg dry wt)⁻¹. In addition this indicated that the *phoS* mutations did not interfere with the derepression of the vegetative enzyme brought about by phosphate starvation as the wild type had a similar specific activity. (This was also true for the *sapA* and *sapB* mutations.)

The pattern of phosphatase synthesis of *phoS* mutants in sporulation conditions was quite distinct from that of *sap* mutants (Fig. 1). The constitutive production of enzyme during vegetative growth is indicated by the high specific activity at the time of resuspension in sporulation medium. The initial drop in specific activity was a consequence of bacterial growth without any increase in total enzyme activity. Subsequently the specific activity did not change greatly; there was, however, considerable enzyme synthesis as there was considerable residual growth in the sporulation medium.

DISCUSSION

Alkaline phosphatase is formed as part of the normal course of events during spore formation. Its formation in sporulation conditions is dependent on the correct expression of four *spoII* loci and at least five *spoO* loci (Piggot & Coote, 1976). We describe here mutations, identifying three new loci, that allow synthesis of phosphatase in sporulation conditions by strains harbouring a variety of *spo* mutations.

For two of the new loci, *sapA* and *sapB*, the effect appears to be specific for the formation of alkaline phosphatase in sporulation conditions as the mutations did not lead to enzyme formation during exponential growth. The *sap* mutations, in themselves, did not prevent the formation of heat-resistant spores (indeed, in a *spo*⁺ background, the *sap* mutations were essentially cryptic). Thus the *sap* mutations are sporulation-associated mutations that are distinct from *spo* mutations. The *sap* loci mapped separately from all known *spo* loci on the *B. subtilis* genetic map (Piggot & Coote, 1976).

The sequence of events during sporulation is likely to be complex after stage III. However, up to stage III, the published data, most notably those of Coote & Mandelstam (1973), were consistent with a single linear dependent sequence of events; synthesis of sporulation alkaline phosphatase was a part of this sequence (see Introduction). In *sapA* and *sapB*

mutants the ability to synthesize sporulation alkaline phosphatase was not dependent on the expression of the various *spo* loci tested, and so was rendered independent of the control of the dependent sequence of events identified by these loci. However, the enzyme was only formed in sporulation conditions and so was still subject to some other form of sporulation control. It can be inferred that, in the wild type, the sequence of sporulation events leading to alkaline phosphatase formation is more complex than a single linear sequence.

Mutations in the third locus, *phoS*, led to enzyme formation during exponential growth as well as in sporulation conditions. Thus it is not possible to say whether the *phoS* mutation disrupts the vegetative or the sporulation-associated control mechanism for phosphatase formation, although the latter is suggested by the close proximity of *phoS* to *sapA* on the genetic map. In contrast to *sapA spo*⁺ mutants, colonies of *phoS spo*⁺ mutants were phenotypically distinguishable from colonies of the wild type.

Miki *et al.* (1965) reported two, genetically distinct, types of phosphatase-constitutive mutant, RI and RII. All the RI mutations were linked by transformation, and so can be considered to lie in a single locus. Mutations of the RI type were later shown to be linked by transduction to *argA* on the *B. subtilis* genetic map (Nukushina & Ikeda, 1969), and the locus was designated *phoR*. The three RII mutations were not linked to this group by transformation. No transformation crosses between the RII mutations were reported, and so they could represent one or several loci. The map position of these mutations is not known. Thus it is possible that one, or more, of the RII mutations may lie in the locus designated here as *phoS*.

There are several reports of mutations that overcome some of the pleiotropic effects of *spoO* mutations in *B. subtilis* (Guespin-Michel, 1971 *a, b*; Ito, 1973; Ito, Mildner & Spizizen, 1971). These studies made use of the increased sensitivity of some stage O mutants to antibiotics and of the ability of certain bacteriophages to replicate only on specific stage O mutants. Different mutations were identified that rendered stage O mutants resistant to antibiotic (*cpsX*, Guespin-Michel, 1971 *b*; *abs*, Ito *et al.*, 1971) or tolerant to bacteriophage (*tol*, Ito, 1973). No map position has been reported for these loci. The secondary mutations also restored, to varying degrees, other functions associated with stage O. The mutations did not restore the ability to sporulate, and there was no indication that they affected properties associated with stages of sporulation after O. In contradistinction the *sap* mutations were originally detected in stage II mutants that were wild type with respect to the various parameters used to isolate and characterize the *cpsX*, *abs* and *tol* mutants. Thus it seems likely that the *sap* loci are distinct from *cpsX*, *abs* and *tol*.

All these secondary mutations can be grouped together conceptually as mutations that overcome some, but not all, of the pleiotropic effects of *spo* mutations. It is to be hoped that a study of the epistasis of such secondary mutations with *spo* mutations will help elucidate the developmental sequence of gene expression during spore formation. However, no clear picture has emerged from the epistasis experiments using *sapA* or *sapB* with different *spo* mutations.

It remains an open question whether alkaline phosphatase is necessary for spore formation, or whether the enzyme is unnecessary and a by-product of some other necessary event. It is clear that formation of alkaline phosphatase in sporulation conditions is not, in itself, adequate to switch on later sporulation-associated events such as the formation of glucose dehydrogenase or dipicolinic acid; expression of these events remains dependent on the correct expression of early *spo* loci whatever the *phoR*, *phoS*, *sapA* or *sapB* allele. The question might be answered if structural gene mutants were available. However, despite intensive search (Grant, 1974; Le Hégarat & Anagnostopoulos, 1969, 1973), these have not been found. The *phoP* mutations, tentatively thought to be in the structural gene (Dubnau, 1970; Nukushina & Ikeda, 1969; Young & Wilson, 1972), have been shown to be control mutations (Grant, 1974; Le Hégarat & Anagnostopoulos, 1973) that do not affect formation of the enzyme during spore formation (Grant, 1974; Ichikawa & Freese, 1974).

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