Sporulation in *Bacillus subtilis* 168. Control of Synthesis of Alkaline Phosphatase

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SUMMARY

Seven mutants which have low activities of vegetative (phosphate-repressible) alkaline phosphatase have been mapped by transduction crosses. These mutations do not affect the production of the alkaline phosphatase associated with sporulation. All the evidence available suggests that the activities produced in phosphate-starvation and during sporulation are attributable to the same protein and derive, presumably, from a common structural gene. It thus appears that alkaline phosphatase has distinct sporulation and vegetative controls and that all the mutations so far examined have been of the regulator type. The position on the chromosome of the structural gene is still in doubt.

INTRODUCTION

Although the synthesis of alkaline phosphatase by *Bacillus subtilis* is repressed when the organism is grown in the presence of inorganic phosphate (Anagnostopoulos, 1960) sporulating cultures of *B. subtilis* 168 produce an alkaline phosphatase which is apparently insensitive to such repression (Warren, 1968). A comparative study of the properties of the 'vegetative' and 'sporulation' alkaline phosphatases led Glenn & Mandelstam (1971) to conclude that they are probably the same protein species. These authors suggested that a common structural gene for the enzyme may be regulated by two distinct control mechanisms, one of which is associated with phosphate starvation while the other is specific to the sporulation process.

In support of this suggestion, Glenn (1971) isolated a number of mutants of *Bacillus subtilis* 168 which were defective in the synthesis of 'vegetative' alkaline phosphatase but were capable of producing the enzyme during sporulation. These mutants, however, sporulated only poorly, and produced variable activities of the 'sporulation' phosphatase.

The present work describes the genetic transfer of the defective 'vegetative' phosphatase character from these and other, similar, mutants into strains isogenic with the wild-type organism. Whereas the strains constructed in this way retained the 'vegetative' phosphatase-negative phenotype of the original isolates, each of them was able to sporulate normally and to synthesize 'sporulation' phosphatase activity equal to that of the wild type. The isolation of these strains provides evidence that synthesis of alkaline phosphatase in *Bacillus subtilis* is under the control of two regulatory systems, one associated with inorganic phosphate starvation of vegetative cells and the other with the participation of this enzyme in the development of the endospore.

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Table 1. *Strains of Bacillus subtilis used in transduction and transformation experiments*

Strain no.	Genotype	Source
BD40	<i>argA3 phe-12</i>	D. Dubnau
GSY289	<i>ura-1 trpC2</i>	C. Anagnostopoulos
GSY334	<i>leu-8</i>	C. Anagnostopoulos
GSY860	<i>argA11 nic-38</i>	C. Anagnostopoulos
MB3	<i>phe-12</i>	P. J. Piggot
MB20	<i>leu-8 trpC2 rif^r tal^r*</i>	P. J. Piggot
MB21	<i>metC3 leu-8 tal^r</i>	P. J. Piggot
MB26	<i>leu-8 phe-12 rif^r tal^r</i>	<i>phe-12</i> transformed into MB20 by congression with <i>trp</i> ⁺

* *tal^r*, resistant to β -thienyl alanine.

METHODS

Organisms. The organism referred to as the 'wild type' in this communication is *Bacillus subtilis* Marburg 168 (*trp C2*), which sporulates normally when supplied with tryptophan or indole. The auxotrophic strains used for transduction and transformation experiments are listed in Table 1. The following 'phosphatase-negative' (*pho*) mutants were studied: NP4. I, NP21. I, NP22. I and NP23. I, which were isolated after mutagenesis of the wild type as described below; P₁₁, R₂R₁ and R₇R₁, also derived from *B. subtilis* 168, which were isolated by Dr A. R. Glenn in this laboratory; Al-1, V12 and V43, which were derivatives of *B. subtilis* 160 and were gifts from Dr T. Miki (Miki, Minami & Ikeda, 1965).

Media. Growth and sporulation media were as described by Coote (1972*a*). In order to ensure complete derepression of synthesis of 'vegetative' alkaline phosphatase in colonies growing on agar plates, the inorganic phosphate concentration of the glucose-glutamate minimal agar medium was lowered to 0.05 mM. This medium is referred to as LP (low phosphate) agar.

Measurement of 'vegetative' alkaline phosphatase activity. Derepression of 'vegetative' alkaline phosphatase synthesis in liquid cultures of *Bacillus subtilis* strains was achieved essentially as described by Glenn & Mandelstam (1971). Their method of assay of the 'vegetative' enzyme was also adopted.

Sporulation and measurement of 'sporulation' alkaline phosphatase activity. Sporulation experiments were performed using the resuspension method of Sterlini & Mandelstam (1969). The production of alkaline phosphatase by a sporulating culture was monitored as follows. Samples (1.0 ml) of the culture were removed at 30 min intervals and centrifuged. The cells were suspended in 1.0 M-diethanolamine-HCl buffer, pH 10.0 (1.0 ml), one drop of toluene was added, and the suspension shaken vigorously for 60 s. One ml of *p*-nitrophenyl phosphate solution (1 mg/ml in the diethanolamine-HCl buffer) was then added and the reaction mixture was incubated at 37 °C for a known time (10 to 15 min). The reaction was stopped by the addition of 2 M-NaOH (1.0 ml) after which the suspension was centrifuged. The extinction of the supernatant solution was measured at 410 nm. One unit of alkaline phosphatase activity is defined as the amount which catalyses the hydrolysis of 1 nmol of *p*-nitrophenyl phosphate/min, and specific activities are expressed as units/mg dry wt of cells.

Isolation of mutants. Mutants were obtained by treatment of the wild type with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). A culture (10 ml) in exponential phase of growth was centrifuged. The cells were suspended in glutamate minimal medium (10 ml) which con-

tained NTG at a final concentration of 125 µg/ml. After this suspension had been shaken at 37 °C for 30 min, samples (0.75 ml) were pipetted into 25 ml volumes of glucose–glutamate minimal medium in 250 ml shake flasks. These were shaken overnight at 37 °C, then diluted and plated on LP agar. After incubation at 37 °C for 2 days, the plates were sprayed at room temperature with a solution of *p*-nitrophenyl phosphate (15 mg/ml in 1.0 M-diethanolamine-HCl buffer, pH 10.0). Colonies with low alkaline phosphatase activity remained white, while normal colonies immediately turned a bright yellow. The white colonies were picked, streaked on glucose–glutamate minimal agar, then restreaked on LP agar to confirm their ‘phosphatase-negative’ (*pho*) character.

Mapping of pho mutants. Transducing lysates of phosphatase mutants were prepared from subcultures of the original isolates using phage PBS-1 as described by Karamata & Gross (1970) and adapted by Coote (1972*b*). The lysates were used to transduce auxotrophic strains, essentially as described by Coote (1972*b*). Transduced cultures and controls were plated on LP agar which lacked the appropriate auxotrophic requirement of the recipient strain. After incubation for 2 days at 37 °C the colonies transduced to prototrophy were screened for their ‘vegetative’ alkaline phosphatase character by spraying the plates with a solution of *p*-nitrophenyl phosphate as described above. A clearer and longer-lasting distinction between the *pho* and *pho*⁺ colonies was obtained if the plates were chilled to 4 °C before they were sprayed. The validity of this method of scoring *pho* and *pho*⁺ inheritance was confirmed by retesting a number of the transductant clones of each type after they had been picked and restreaked to single colonies.

In the three-factor crosses, MB26 (*leu-8 pho*⁺ *phe-12*) was transduced with the lysates of the phosphatase mutants (*leu*⁺ *pho phe*⁺). *leu*⁺ transductants were selected and transferred to LP plates with and without phenylalanine supplement. The inheritance of the *phe* character was then determined by inspection, and that of the *pho* character by spraying the phenylalanine-supplemented plates with *p*-nitrophenyl phosphate solution as described above.

Preparation of ‘isogenic’ strains. Strain MB21 (*metC3 leu-8 tal*⁺), isogenic with the wild type except in its known genetic markers, was transformed with DNA from alkaline phosphatase mutants by the method of Ephrati-Elizur (1968) as used by Piggot (1973). *leu*⁺ transformants were selected. The need for a counter-selection against growth of donor bacteria in this transformation procedure was satisfied by the auxotrophic requirements of the phosphatase mutants (tryptophan or arginine). A small number of the *leu*⁺ transformants inherited the *pho* character by congression.

RESULTS

‘Phosphatase-negative’ (pho) mutants. When grown on LP agar, all ten ‘phosphatase-negative’ mutants used in the present study were apparently devoid of alkaline phosphatase activity, but experiments with liquid cultures showed that, upon removal of inorganic phosphate from the medium, some alkaline phosphatase activity could be detected. The maximum activity produced was usually about 1 to 3 % of that of a wild-type control culture, and although this varied from one experiment to another with any given mutant, activities greater than 5 % of that produced by the control culture were never recorded.

Genetic mapping of the pho mutants. The seven *pho* mutants derived from *Bacillus subtilis* 168 were mapped by two- and three-factor transduction crosses. The results in Table 2 show that all 7 mutations were linked by transduction to *argA11*, *leu-8* and *phe-12*, but not to *ura-1*. As *leu* is only weakly linked to *argA* (approximately 20 % cotransduction; Dubnau

Table 2. Two-factor transduction crosses to determine linkage of *pho* mutations to the markers *ura-I*, *argAII*, *leu-8* and *phe-12*

Mutant	Linkage to selected markers*			
	<i>ura-I</i>	<i>argAII</i>	<i>leu-8</i>	<i>phe-12</i>
NP4. I	0 (0/364)	75 (66/88)	41 (79/195)	21 (154/741)
NP21. I	0 (0/210)	n.t.	44 (136/312)	22 (127/579)
NP22. I	0 (0/269)	56 (106/191)	27 (341/1250)	17 (150/883)
NP23. I	0 (0/260)	46 (33/72)	40 (129/319)	22 (74/342)
P ₁₁	0 (0/156)	54 (368/685)	47 (46/98)	21 (159/743)
R ₂ R ₁	0 (0/308)	60 (357/599)	51 (213/420)	23 (201/879)
R ₇ R ₁	0 (0/200)	67 (139/209)	46 (188/405)	21 (179/838)

* PBS-1 lysates were prepared on the *pho* mutants (*ura*⁺, *arg*⁺, *leu*⁺ and *phe*⁺), and used to transduce GSY289 to *ura*⁺, GSY860 to *arg*⁺, GSY334 to *leu*⁺, and MB3 to *phe*⁺. Linkage is shown as percentage cotransduction with the number of transductants in parentheses (*pho*/(*pho*⁺ + *pho*)). n.t. = Not tested.

Table 3. Three-factor transduction crosses to determine the position of *pho* mutations with respect to the *leu-8* and *phe-12* markers. Recipient genotype (MB26): *leu-8 pho*⁺ *phe-12* (ooo). Donor genotype: *leu*⁺ *pho phe*⁺ (III)

Selected marker	Donor		Number of transductants in the recombinant classes				Possible order	No. of transductants in class requiring 4 cross-overs	Suggested order
	Unselected markers		100	101	110	111			
<i>leu</i> ⁺	<i>pho</i> NP4. I	<i>phe</i> ⁺	7	53	22	28	<i>leu-pho-phe</i> <i>leu-phe-pho</i> <i>pho-leu-phe</i>	53 22 —*	<i>pho-leu-phe</i>
<i>leu</i> ⁺	<i>pho</i> NP21. I	<i>phe</i> ⁺	18	60	7	25	<i>leu-pho-phe</i> <i>leu-phe-pho</i> <i>pho-leu-phe</i>	60 7 —*	<i>pho</i> { <i>leu</i> } { <i>phe</i> }
<i>leu</i> ⁺	<i>pho</i> NP22. I	<i>phe</i> ⁺	31	59	9	9	<i>leu-pho-phe</i> <i>leu-phe-pho</i> <i>pho-leu-phe</i>	59 9 —*	<i>pho</i> { <i>leu</i> } { <i>phe</i> }
<i>leu</i> ⁺	<i>pho</i> NP23. I	<i>phe</i> ⁺	21	48	20	19	<i>leu-pho-phe</i> <i>leu-phe-pho</i> <i>pho-leu-phe</i>	48 20 —*	<i>pho-leu-phe</i>
<i>leu</i> ⁺	<i>pho</i> P ₁₁	<i>phe</i> ⁺	13	61	14	24	<i>leu-pho-phe</i> <i>leu-phe-pho</i> <i>pho-leu-phe</i>	61 14 —*	<i>pho-leu-phe</i>
<i>leu</i> ⁺	<i>pho</i> R ₂ R ₁	<i>phe</i> ⁺	15	65	17	15	<i>leu-pho-phe</i> <i>leu-phe-pho</i> <i>pho-leu-phe</i>	65 17 —*	<i>pho-leu-phe</i>
<i>leu</i> ⁺	<i>pho</i> R ₇ R ₁	<i>phe</i> ⁺	17	66	13	15	<i>leu-pho-phe</i> <i>leu-phe-pho</i> <i>pho-leu-phe</i>	66 13 —*	<i>pho-leu-phe</i>

* Multiple cross-overs are undetectable from the possible order *pho-leu-phe*.

et al. 1967), the percentage cotransduction figures suggest the order *argA-pho-leu-phe*, and this is confirmed by the results of the three-factor crosses (Table 3).

Linkage to *leu-8* of the *pho* mutations carried by the 160-derived strains v12 and v43 was also established, although poor transducing activity of lysates prepared from these strains precluded a meaningful estimation of the linkage distance. Linkage of v12 and v43 to the *argA* marker can be deduced from the results of Nukushina & Ikeda (1969) with sp-25, another *pho* mutant linked by transformation to both v12 and v43 (Miki *et al.* 1965).

Table 4. *Sporulation of isogenic strains carrying pho mutations*

Bacteria from an exponential culture in hydrolysed casein medium were centrifuged and resuspended in a simple defined medium to induce sporulation (Sterlini & Mandelstam, 1969). Samples were taken at intervals for estimation of phosphatase activity which was usually at a maximum after 4 h.

Mutant*	'Sporulation' phosphatase†		Refractility‡	
	168 (control)	<i>pho</i> strain	168 (control)	<i>pho</i> strain
NP4. I	44.0	45.0	70	65
NP22. I	—	50.0	—	95
P ₁₁	33.6	32.5	74	78
R ₂ R ₁	51.6	43.5	80	70
R ₇ R ₁	42.9	38.4	80	80
Al-I	37.5	29.2	65	60
VI2	31.8	25.6	65	65
V43	41.0	40.4	75	67

* Original isolate used to construct isogenic strain.

† Maximum activity (units/mg dry wt of cells) of alkaline phosphatase during sporulation.

‡ Percentage of cells containing refractile spores after 7 h in resuspension medium.

These findings are in accord with the work of Le Hegarat & Anagnostopoulos (1969) who mapped four alkaline phosphatase mutants by transduction and transformation; these mutants were similar in properties to those used in the present study (C. Anagnostopoulos, personal communication), and were located in a single gene between the *argA* and *leu* markers.

Sporulation of the pho mutants. The mutations from eight of the ten *pho* mutants were transferred by transformation crosses to recipient strains which were isogenic with the wild type except for known genetic markers. Each of the constructed *pho* strains was then tested for its ability to sporulate in a resuspension culture. Wild type was included as a control in each experiment, and the alkaline phosphatase activities in both cultures were assayed. The results of these experiments are presented in Table 4. After 7 h in the resuspension medium the culture of each *pho* strain was subcultured and subsequently tested for its ability to produce the 'vegetative' alkaline phosphatase. In all cases the 'vegetative' *pho* phenotype was retained.

These results clearly show that the *pho* mutations carried by these strains have no effect on sporulation or on the production of 'sporulation' alkaline phosphatase. This conclusion is supported by results of similar experiments on the original isolates of the two remaining mutants, NP21. I and NP23. I. Both mutants sporulated as well as the wild type, and synthesized normal, wild-type alkaline phosphatase activities during sporulation.

It should be mentioned that, apart from NP21. I and NP23. I, the original isolates of these *pho* mutants sporulated only very poorly in resuspension experiments; the yield of refractile spores was never greater than 10 %, and the production of 'sporulation' alkaline phosphatase was low and variable. It is likely that this behaviour was the result of multiple mutation, particularly in the case of the mutants induced by NTG mutagenesis (Guerola, Ingraham & Cerdá-Olmedo, 1971). This emphasizes the importance of using isogenic strains.

DISCUSSION

Seven mutants have been isolated from *Bacillus subtilis* 168 which produce very low activities of alkaline phosphatase under normal conditions for derepression of synthesis of the enzyme during vegetative growth. These mutants have been mapped by transduction,

and the linkage data indicate that all seven mutations lie in a region between the *argA* and *leu* markers on the chromosome. It has been demonstrated that strains carrying these mutations sporulate normally and produce the same level of 'sporulation' alkaline phosphatase as does the wild-type control. If, as seems likely from the work of Glenn & Mandelstam (1971), the 'vegetative' and 'sporulation' alkaline phosphatases are coded by a common structural gene, the present results imply that the mutations mapped in the *argA-leu* region are in a regulatory gene (or genes) specifically concerned with control of synthesis of the 'vegetative' enzyme. Control of synthesis of the 'sporulation' phosphatase is thus an independent, or at least partly independent, process with its own control genes.

In their genetic studies on ten *pho* mutants derived from *Bacillus subtilis* 160, Miki *et al.* (1965) found that, although all ten mutations were linked by transformation, they could be divided into two clusters, P-I and P-II. Indirect evidence suggested that P-I represented the structural gene and P-II a regulatory gene for alkaline phosphatase synthesis, and this interpretation has been adopted in recent reviews of the genetics of *B. subtilis* (Dubnau, 1970; Young & Wilson, 1972). As has been described above, three of the mutants used by Miki *et al.* (1965) have been studied in the present work, viz Al-I and V43 from the P-I region, and V12 from the P-II region. Our results show that the *pho* mutations in these three strains have no effect on the production of normal alkaline phosphatase activity during sporulation. In view of the findings of Glenn & Mandelstam (1971), this implies that these mutations too are in a regulatory gene (or genes) rather than a structural gene. It is concluded that there are at least two 'vegetative' regulation sites as well as a 'sporulation' regulation site and that the position of the structural gene for alkaline phosphatase on the chromosome of *B. subtilis* is still undetermined.

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