Aust. J. Biol. Sci., 1975, **28**, 323–30

Alkaline Phosphatase Mutants of *Bacillus subtilis*

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Abstract

Alkaline phosphatases from vegetative and sporulating cells of *B. subtilis* have been shown previously to be identical in all criteria examined. Despite this, 15 mutants producing low levels of the phosphatase during phosphate starvation of vegetative cells have been shown to produce high levels of the sporulation-specific alkaline phosphatase. It has been shown by immunochemical means that seven of these mutants when starved of phosphate produce low levels of normal wild-type enzyme. The sporulation form of the enzyme from one mutant (P-100) has been shown to be identical with the phosphatases from vegetative and sporulating cells of the wild type. It is proposed that all the mutants have regulatory defects in the control of the alkaline phosphatase from vegetative cells but nevertheless retain an intact structural gene for the enzyme and the control system for the phosphatase during sporulation.

Introduction

It has been shown that *Bacillus subtilis* possesses an alkaline phosphatase which, like the enzyme from *Escherichia coli* (Horiuchi *et al.* 1959), is repressible by inorganic phosphate (Anagnostopoulos 1960; Takeda and Tsugita 1967). However, an alkaline phosphatase is produced during sporulation of *B. subtilis*, albeit in much lower amounts, even in the presence of concentrations of inorganic phosphate which completely repress the vegetative (i.e. phosphate repressible) enzyme (Warren 1968). Examination of the morphological and biochemical properties of asporogenous mutants suggested that this increase in phosphatase activity during spore formation was sporulation-specific and that it might be associated with stage II (spore septum formation) of the sporulation process (Waites *et al.* 1970; Coote 1972). Cytochemical studies have provided additional evidence for linking the appearance of alkaline phosphatase during sporulation with stage II (Glenn 1971). Early blocked sporulation mutants (i.e. all stage 0 and stage I and most of stage II) fail to produce alkaline phosphatase (Waites *et al.* 1970; Coote 1972) although they produce normal levels of phosphatase when starved of phosphate in the vegetative state (Glenn 1971).

Although the phosphatases from vegetative and sporulating cells differ in phosphate repressibility the two activities were indistinguishable when compared by a large number of criteria, including localization, solubility properties, salt activation, pH optimum, K_m and K_i, molecular weight, electrophoretic mobility, thermal activation and inactivation, and immunochemical cross-reactivity (Glenn 1971; Glenn and Mandelstam 1971). It was suggested, therefore, that the alkaline phosphatase activities from vegetative and sporulating cells resided in a single protein species and that the differences in regulation were the result of a complex regulation system.

In order to examine further the genetic relationship between the phosphatases from vegetative and sporulating cells, mutants were isolated which were defective in alkaline phosphatase production in the vegetative state. This paper describes the properties of these phosphatase mutants.

Materials and Methods

Organism

Bacillus subtilis (Marburg) is an indole or tryptophan auxotroph which sporulates normally. Alkaline phosphatase negative mutant S31 was obtained from Dr J. Spizizen and the negative and constitutive strains BC-7, A1-1, Sp-22, V-11, Sp-25, V-43, V-12 and Sg-13 derived from *B. subtilis* 160 were obtained from Professor Y. Ikeda.

Isolation of Phosphatase Mutants

Wild-type *B. subtilis* was grown in a casamino acid growth medium (Sterlini and Mandelstam 1969) from a spore inoculum to a density of 0·1–0·15 mg dry weight/ml, harvested and resuspended in a glucose–glutamate medium (Sterlini and Mandelstam 1969) containing *N'*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (125 μg/ml) (Adelberg *et al.* 1965). The culture was then shaken for 30 min after which time 1·5-ml samples were inoculated into ten 50-ml portions of glucose–glutamate medium. The cultures were grown at 37°C until turbid, and then diluted and plated onto glucose–glutamate agar plates. After incubation for 48 h at 37°C the plates were sprayed with *p*-nitrophenyl phosphate (15 mg/ml in diethanolamine hydrochloride, pH 10); wild-type or constitutive colonies gave an intense yellow colour within seconds whereas negative mutant colonies remained white or turned yellow only slowly.

Heat treatment at 62°C was also employed to isolated phosphatase mutants (Rogolsky 1969). Wild-type cells were grown in the casamino acid growth medium to 0·1 mg dry weight/ml and then heated at 62°C for 20 min. After heat treatment the culture was divided and the portions allowed to grow at 37°C until they reached a cell density of 0·4–0·5 mg dry weight/ml when they were diluted and plated onto glucose–glutamate agar plates. Mutant colonies were identified as before.

Culture and Procedure for Sporulation

Cells were grown at 37°C in the hydrolysed casein medium to a density of 0·2–0·3 mg dry weight/ml and sporulation induced by resuspending them in the same volume of warm glutamate salts 'resuspension' medium (Sterlini and Mandelstam 1969). After 7–8 h the wild type normally gave 70–80% refractile spores.

Nomenclature

The point of initiation to sporulation t_0 is the time of resuspension from the growth medium: t_1, t_2 etc. represent hourly intervals thereafter. Stages 0–VI are the recognized cytological stages of spore formation (Ryter 1965).

Depression of Alkaline Phosphatase in Vegetative Cells

Cells were cultured in the phosphate-limited Bactopeptone medium as described previously (Glenn and Mandelstam 1971).

Enzyme Assay

Alkaline phosphatase activity was measured according to the method described by Glenn and Mandelstam (1971). Units of vegetative alkaline phosphatase are expressed as μmoles *p*-nitrophenol released per minute; units of sporulation phosphatase are expressed as nmoles of *p*-nitrophenol released per minute.

Bacterial Density

This was measured spectrophotometrically using a calibration curve relating E_{600} to dry weight.

Sephadex G100 Chromatography

Sephadex G100 columns were prepared by the method of Andrews (1964). Proteins were eluted with 0·2M $MgCl_2$ in tris–HCl buffer (10 mM, pH 7·4). The flow rate was 15–20 ml/h and 10-ml fractions were collected. The void volume of the column (78 by 3·4 cm) was estimated by the elution of Blue Dextran G2000 and was 160 ml.

Precipitation with Antiserum

Antiserum was prepared against purified alkaline phosphatase as described previously (Glenn and Mandelstam 1971). Diffusion experiments were carried out as described by Ouchterlony (1958) except that the agar concentration was 0·15% and 0·2M $MgCl_2$ was added to the agar in order to maintain the phosphatase in solution. A method similar to that of Preer and Preer (1959) was used to estimate the amount of cross-reacting material. When serial dilutions of wild-type enzyme of known activity were allowed to diffuse against a constant amount of antiserum, the plot of the position of the precipitin band relative to the antigen origin against enzyme concentration gave a straight-line relationship. The relative amount of cross-reacting material in an extract was estimated by the position of the precipitation band in the agar as compared with the position of the band formed by a standard solution of the wild-type enzyme. Enzyme–antibody precipitin bands were stained with α-naphthyl acid phosphate and Fast Blue RR salt (Sigma Chemical Co. Ltd., St. Louis, Mo., U.S.A.); the phosphatase antibody does not inactivate enzyme activity.

Fig. 1

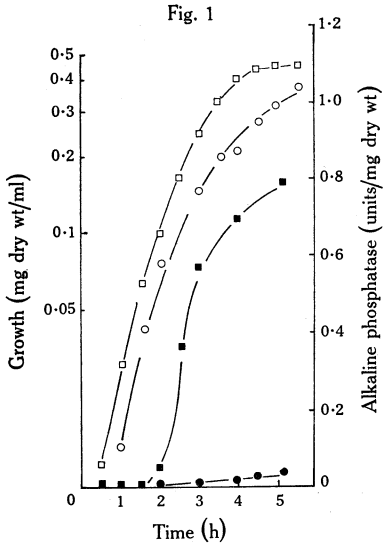

Fig. 2

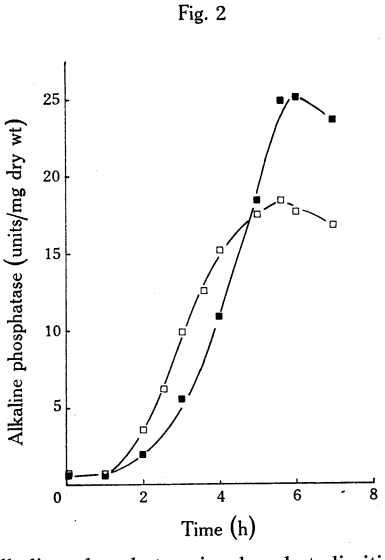

Fig. 1. Growth and formation of vegetative alkaline phosphatase in phosphate-limiting Bactopeptone medium. Bactopeptone (50 ml) was inoculated with 0·1 ml of overnight Penassay broth culture of wild type 168 and phosphatase mutant S-31 to give an initial density of 8 μg dry weight/ml. Samples were taken at intervals for extinction and enzyme assay. Alkaline phosphatase activity in wild type (■) and S-31 (●). Growth in wild type (□) and S-31 (○).

Fig. 2. The appearance of sporulation alkaline phosphatase in wild type (■) and phosphatase mutant P-100 (□). Bacteria were resuspended for sporulation and at intervals samples taken for alkaline phosphatase assay.

Results

Growth Characteristics and the Production of Alkaline Phosphatase in Vegetative Cells of Phosphatase Mutants

The isolation procedure selected mutants deficient in alkaline phosphatase in the vegetative state; because of the pleiotropic effects of mutations in the sporulation

process it is not possible to select for mutants specifically lacking the sporulation phosphatase. Both nitrosoguanidine and heat shock mutagenesis gave rise to mutants lacking alkaline phosphatase. All the mutants grew on minimal glucose–glutamate or lactate–glutamate media at rates comparable to the wild type. Similarly, in the phosphate-limiting Bactopeptone–salts medium the mutants grew with a mean generation time of 34 min similar to the wild type. The phosphatase mutants appear, therefore, to be unimpaired in normal vegetative growth. When starved of phosphate in the Bactopeptone medium all of the mutants were found to be deficient in alkaline phosphatase (3–5% of the wild-type levels of the enzyme) rather than totally lacking the enzyme (Fig. 1 and Table 1).

Table 1. Production of vegetative and sporulation alkaline phosphatase by phosphatase mutants
Bacteria were either grown in the phosphate-limited Bactopeptone salts medium to the stationary phase, when the cultures were assayed for vegetative alkaline phosphatase activity, or were transferred from the growth medium at mid-log phase to the resuspension medium to induce sporulation and samples taken at hourly intervals for measurement of extinction and sporulation alkaline phosphatase activity. The values for both the vegetative and sporulation phosphatases are given as a percentage of the wild-type levels, those for the sporulation enzyme being recorded at 5 h and expressed as a percentage of the wild-type levels at that time. The 100% value in the wild type for vegetative and sporulation enzyme was 0·45 and 34·4 units per millilitre of culture medium respectively

Strain	Alkaline phosphatase levels		Strain	Alkaline phosphatase levels	
	Vegetative	Sporulation		Vegetative	Sporulation
168	100	100	Al-1	2·7	49
P-100	5·1	69	Sp-22	3·9	74
P-103	5·0	47	V-11	4·0	66
P-104	5·0	30	Sp-25	2·1	25
P-105	4·1	13	V-43	5·1	58
P-8	3·8	95	V-12	3·8	56
P-9	5·2	34	Sg-13	4·3	81
P-11	4·0	26	S-31	4·2	62

The mutants were examined for production of cross-reacting material to antiserum prepared against purified wild-type phosphatase. Mutant cells were cultured overnight in Bactopeptone, harvested by centrifugation and the resulting cell pellet (100–150 mg dry weight) extracted with 1·0 ml tris–HCl buffer (10 mM, pH 7·4) containing 1·0M $MgCl_2$. It has been shown previously that this ionic elution procedure extracts enzyme from the wild type (Glenn and Mandelstam 1971). Cells were removed by centrifugation and the supernatant salt wash (75 μl) used in Ouchterlony double diffusion tests against antiserum (40 μl). None of the phosphatase mutants produced any cross-reacting material when tested in this manner, although the wild type yielded a material which gave a heavy precipitin band (75 μl extract, 7·5 enzyme units). Material similarly extracted from a constitutive strain BC-7 cross-reacted with complete identity when tested against the antiserum (40 μl). All attempts to reveal cross-reacting material in the supernatants of phosphate-starved cultures of the phosphatase mutants failed.

When salt-eluted material from large-scale cultures of P-100, P-103, P-104, P-105, P-8 and P-9 was concentrated approximately 20-fold by vacuum dialysis, and then examined in double diffusion tests (75 μl, 7·5 units of enzyme activity) all gave rise to precipitin bands. The precipitin lines from the mutants cross-reacted with complete

identity with each other and with the enzyme derived from the wild type; these precipitin lines stained positively for alkaline phosphatase when the diffusion plates were flooded with α-naphthyl acid phosphate and Fast Blue RR salt in borax (0·6%).

The relative amount of cross-reacting material in the salt-extracted material from the large-scale vegetative cultures of the phosphatase mutants was examined in diffusion tests by the method of Preer and Preer (1959). Serial dilutions of the salt-extracted and concentrated material from the mutants, of known enzyme activity, were assayed against a constant concentration of antiserum (40 μl). It was found that in all the mutants the specific enzyme activity per unit of cross-reacting material was very similar to that of the wild type.

The concentrated material from P-100, P-104, P-105, P-8 and P-9 was examined for heat stability at 65°C. Heat stability is often radically altered by mutation and this provides a means of testing for changes in structural genes. The protein concentration of the extracts was adjusted to 5·0 mg/ml with bovine plasma albumin. The extracts were heated at 65°C and samples taken at intervals and assayed for residual activity. All of the mutant enzyme samples had $t_{\frac{1}{2}}$ of 26–28 min identical to the wild-type enzyme (Glenn and Mandelstam 1971).

Production of Alkaline Phosphatase during Sporulation of Phosphatase Mutants

Since previous work had shown that the alkaline phosphatases from vegetative and sporulating cells were indistinguishable and were possibly the same gene product, it was of considerable interest to examine the phosphatase-deficient mutants for the production of the sporulation enzyme.

Mutant cells were grown to mid-log phase in the casein hydrolysate medium and induced to sporulate by resuspension in the glutamate salts medium (Sterlini and Mandelstam 1969). Samples were taken at intervals to assay for alkaline phosphatase activity. A typical experiment showing the appearance of sporulation alkaline phosphatase activity in the wild type and mutant P-100 is shown in Fig. 2. The levels of alkaline phosphatase are somewhat variable even in the wild type and the results shown in Table 1 are the average of three or more separate experiments. The mutants varied in the level of phosphatase they produced, e.g. P-105 produced only about 20% of the wild-type level whereas P-8 produced virtually wild-type levels; other mutants gave intermediate levels of the enzyme (Table 1). Nevertheless, the level of the alkaline phosphatase produced by these mutants under sporulation conditions although less, is very much closer to wild-type levels than under conditions of phosphate starvation in the vegetative state (Table 1).

The alkaline phosphatase from sporulating cells of one of these mutants, P-100, has been partially purified and its properties compared with the alkaline phosphatases from vegetative and sporulating cells of the wild type. Phosphatase from resuspension sporulation cultures of P-100 at t_5 was extracted by ionic elution with tris–HCl buffer containing 1·0M $MgCl_2$. The material was then concentrated by dialysis against solid sucrose. The molecular size of the phosphatase from P-100 was estimated by gel filtration on Sephadex G100 using cytochrome *c*, trypsin inhibitor, ovalbumin, bovine plasma albumin, *E. coli* alkaline phosphatase, and γ-globulin as protein standards. The phosphatase from sporulating cells of P-100 had a molecular weight of about 69000 (Fig. 3*a*) identical to that observed previously for the sporulation and vegetative enzymes from the wild type (Glenn and Mandelstam 1971). Phos-

phatase from sporulating cells of P-100 was examined for its heat stability at 65°C; the protein concentration was adjusted to 5 mg/ml with bovine plasma albumin and samples taken at intervals to assay for residual enzyme activity. The $t_{\frac{1}{2}}$ of the alkaline phosphatase from sporulating cells of P-100 was 26 min (Fig. 3*b*), very similar to the value of 26–27 min recorded for the enzyme from vegetative and sporulating cells of the wild type (Glenn and Mandelstam 1971) and for the material prepared from the phosphate-starved phosphatase mutants (see earlier).

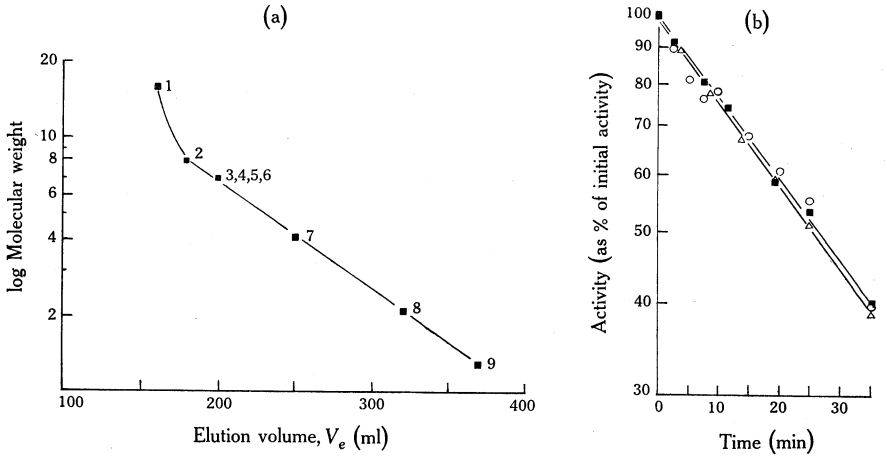

Fig. 3. (*a*) Estimation of the molecular weight of the sporulation alkaline phosphatase from P-100 on Sephadex G100. The column (78 by 3·4 cm) was calibrated with proteins of known molecular weight: 1, γ-globulin; 2, *E. coli* alkaline phosphatase; 3, bovine plasma albumin; 4, wild-type vegetative alkaline phosphatase; 5, wild-type sporulation alkaline phosphatase; 6, P-100 sporulation alkaline phosphatase; 7, ovalbumin; 8, trypsin inhibitor; 9, cytochrome *c*. Vegetative and sporulation phosphatase from both wild type and P-100 co-eluted with bovine plasma albumin (3). (*b*) Thermal inactivation of vegetative and sporulation phosphatase from wild type and P-100 at 65°C in tris–HCl buffer (10 mM pH 7·4) containing 0·2M $MgCl_2$. ■ Wild-type vegetative phosphatase. ○ Wild-type sporulation phosphatase. △ P-100 sporulation phosphatase.

Ouchterlony double diffusion tests on the partially purified material from P-100 showed that it cross-reacted with the antiserum prepared against the purified enzyme The precipitin lines, using wild-type enzymes and material extracted and concentrated from phosphate-starved vegetative cells of P-100 as control, were continuous, non-spurred and stained positively for alkaline phosphatase.

These experiments provide strong evidence that the alkaline phosphatase produced during sporulation of phosphatase mutants is identical to the enzymes found in vegetative and sporulating cells of the wild type and is not some totally different enzyme.

Discussion

The synthesis of alkaline phosphatase during spore formation in mutants deficient in the vegetative form of the enzyme can be explained in two ways. Firstly, there may be distinct structural genes for the 'sporulation' and 'vegetative' enzymes; since the two phosphatase activities appear to be identical, it would suggest that there was structural gene duplication for the alkaline phosphatases. The regulator elements for these separate genes would also be distinct, and this separation would explain

the differences in control. The second explanation of these observations, would suggest that since the two enzyme activities are identical, they are most likely to be the product of the same gene. The control of the phosphatase, however, would be regulated by distinct control elements in the vegetative and sporulating state. Such a suggestion would, of course, imply that all the mutants used in this study are regulatory rather than structural mutants. Despite an extensive search by several workers (Glenn 1971; Grant 1974; Le Hegarat and Anagnostopoulos 1974) no phosphatase mutant of *B. subtilis* has yet been isolated that produces a cross-reacting material, i.e. mutant enzyme with a low specific activity per unit cross-reacting material. Although a number of phosphatase mutants (including some of those used in this study) have been mapped on the *B. subtilis* chromosome, the location of the structural gene is unknown (Miki *et al.* 1965; Le Hegarat and Anagnostpoulos 1969; Grant 1974).

The immunological analysis by the Preer and Preer (1959) technique of seven phosphatase-deficient mutants which showed that the specific activity per unit of cross-reacting material was similar to the wild type in all cases provides strong evidence that all the mutants are producing very low levels of normal wild-type enzyme. This contention is further supported by the finding that the heat stability of the enzyme from vegetative cells of several of the mutants was identical to that of the wild-type enzyme. It seems reasonable to suggest, therefore, that all the mutants used in this study have some defect in the control of the alkaline phosphatase in the vegetative state, but that they retain an intact structural gene for the enzyme and also the control system for switching on the sporulation specific phosphatase. A similar suggestion has recently been advanced by Grant (1974).

Although there is strong evidence that the phosphatase activities of vegetative and sporulating cells reside in a single protein species (Glenn and Mandelstam 1971) there are apparent differences in the control of the two forms. One suggestion for the apparent differences in control is that the phosphatase is produced during spore formation only when the spore protoplast is produced inside the mother cell (i.e. stage III of spore formation), and this structure becomes phosphate-starved, although there is excess phosphate in the medium. Studies on the phosphate metabolism of sporulating cells by Nelson and Kornberg (1970) and Ichikawa and Freese (1974) have provided evidence that phosphate depletion in sporulating cells is not the inducing mechanism for alkaline phosphatase during spore formation. Additional evidence for rejecting phosphate starvation as the inducing mechanism has come from cytochemical studies which have shown that phosphatase is found in stage II cells, i.e. prior to spore protoplast formation (Glenn 1971). The isolation of a series of mutants which appear to be defective in the ability to respond to phosphate starvation, yet are able to produce normal, or near normal, levels of alkaline phosphatase during sporulation, provides a compelling argument for believing that the spore enzyme is controlled by some mechanism distinct from phosphate starvation and which is sporulation specific.

At this stage there seems no obvious explanation as to why mutants in the structural gene for alkaline phosphatase are difficult to isolate. The isolation of a mutant positive for cross-reacting material would greatly facilitate the study of the control of alkaline phosphatase and would allow further investigation into the relationship between the enzymes from vegetative and sporulating cells.

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Manuscript received 27 September 1974