Bacteriophage PBSX-induced Deletion Mutants of *Bacillus subtilis* 168 Constitutive for Alkaline Phosphatase

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Mutants of *Bacillus subtilis* with deletions extending from the PBSX prophage, and in some cases removing *pro(AB)* and *metC*, have been found to be constitutive for vegetatively synthesized alkaline phosphatase. Such deletions were isolated by selecting for heat-resistant derivatives of a strain carrying a *xhi-1479* mutation causing heat-inducibility of the defective phage PBSX. These deletions remove the *phoS* gene, a regulatory gene for alkaline phosphatase; it is concluded that the *phoS* gene product exerts negative control on alkaline phosphatase synthesis. Deletion mapping, combined with previously published linkage data, indicates a gene order of PBSX–*phoS*–*pro(AB)*–*metC*.

INTRODUCTION

Alkaline phosphatase synthesis by *Bacillus subtilis* is repressed by inorganic phosphate during exponential growth (Anagnostopoulos, 1960). Mutations in three loci, *phoR* (Le Hégarat & Anagnostopoulos, 1969; Miki *et al.*, 1965; Nukushina & Ikeda, 1969), *phoS* (Piggot & Taylor, 1977) and *phoT* (Piggot *et al.*, 1981), result in constitutive enzyme formation, whereas mutation in a fourth gene, *phoP*, causes alkaline phosphatase to be completely repressed (Le Hégarat & Anagnostopoulos, 1969; Miki *et al.*, 1965; Nukushina & Ikeda, 1969). Other controls apparently operate during sporulation where the enzyme is formed in the presence of inorganic phosphate. Mutation in any one of 14 *spo* loci prevents this synthesis whilst mutation in *sapA* or *sapB* overcomes the effects of the *spo* mutations (Piggot & Coote, 1976; Piggot & Taylor, 1977; P. J. Piggot, unpublished observations). Nothing is yet known, however, of how any of these loci exert their control on the synthesis of alkaline phosphatase.

One very good criterion for determining whether a gene is regulated by positive or negative control is the characterization of deletions of the regulatory gene (see, for example, Beckwith & Rossow, 1974). Constitutive expression should result from deletion of a regulatory gene exerting negative control, whereas loss of expression should result from the deletion of a regulatory gene operating by positive control. Such a method has been used to demonstrate positive control of alkaline phosphatase synthesis by the *phoB* gene product in *Escherichia coli* (Brickman & Beckwith, 1975). Until recently, however, this type of analysis has not been possible in *B. subtilis*, because no method has been available to delete parts of the chromosome. This has been remedied by the isolation of prophage mutations causing heat-inducibility of the defective bacteriophage PBSX (*xhi-1479*) (Buxton, 1976), and of bacteriophage SP β (*c2*) (Rosenthal *et al.*, 1979). Thus, it is possible to generate deletions in the region adjacent to the PBSX prophage by selecting for heat-resistant derivatives of the *xhi* mutant (Buxton, 1980). Coincidentally, two of the loci regulating alkaline phosphatase synthesis, *phoS* and *sapA*, map in the region of the chromosome adjacent to the PBSX prophage (Buxton, 1980; Piggot & Taylor, 1977). We have therefore generated deletions in this region and we show that these mutants produce alkaline phosphatase constitutively in vegetative growth.

Table 1. *Strains of Bacillus subtilis* used

Strain	Genotype	Origin or reference
RB1037	<i>pyrX</i> * <i>xhi-1479</i> † <i>xki-1479</i> ‡	Buxton (1980)
RB1081	<i>pyrX</i> Δ(PBSX- <i>phoS</i> - <i>pro</i> (AB))	Heat ^r derivative of RB1037. Buxton (1980)
RB1084	<i>pyrX</i> Δ(PBSX- <i>phoS</i> - <i>pro</i> (AB)- <i>metC</i>)	Heat ^r derivative of RB1037. Buxton (1980)
RB1180	<i>pyrX</i> <i>xhi-1479</i> <i>xki-1479</i> <i>rif-2</i> <i>spoIIA69</i>	DNA.SL652 × RB1037 → Rif ^r
RB1183	<i>pyrX</i> Δ(PBSX), or <i>xhi</i> ⁺	Heat ^r derivative of RB1037
RB1184	<i>pyrX</i> Δ(PBSX), or <i>xhi</i> ⁺ , <i>rif-2</i> <i>spoIIA69</i>	Heat ^r derivative of RB1180
RB1190	<i>pyrX</i> Δ(PBSX- <i>phoS</i> - <i>rif-2</i> <i>spoIIA69</i>)	Heat ^r derivative of RB1180
RB1193	<i>pyrX</i> Δ(PBSX- <i>phoS</i> - <i>pro</i> (AB)- <i>metC</i>) <i>rif-2</i> <i>spoIIA69</i>	Heat ^r derivative of RB1180
RB1388	<i>trpC2</i>	M. M. McConnell
SL321	<i>sapB2</i> <i>leuA8</i> <i>rif-2</i> <i>spoIIG55</i>	Previously designated 300.16. Piggot & Taylor (1977)
SL340	<i>phoS5</i> <i>rif-2</i> <i>spoIIA69</i>	Previously designated 305.1. Piggot & Taylor (1977)
SL344	<i>phoS5</i> <i>leuA8</i> <i>tal-1</i> §	Previously designated 305.7. Piggot & Taylor (1977)
SL345	<i>phoS5</i> <i>leuA8</i> <i>rif-2</i> <i>spoIIE64</i>	DNA.64.1 (Piggot & Taylor, 1977) × SL344 → Rif ^r
SL349	<i>sapA6</i> <i>leuA8</i> <i>tal-1</i>	Previously designated 306.7. Piggot & Taylor (1977)
SL351	<i>sapA6</i> <i>leuA8</i> <i>tal-1</i> <i>rif-2</i> <i>spoIIE64</i>	DNA.64.1 × SL349 → Rif ^r
SL652	<i>purB6</i> <i>rif-2</i> <i>spoIIA69</i>	Previously designated 69.3. Piggot & Taylor (1977)
SL1053	<i>pyrX</i> Δ(PBSX- <i>phoS</i> - <i>pro</i> (AB)- <i>metC</i>) <i>rif-2</i> <i>spoIIA69</i>	DNA.SL652 × RB1084 → Rif ^r
SL1059	<i>pyrX</i> Δ(PBSX- <i>phoS</i>) <i>rif-2</i> <i>spo</i> ⁺	DNA.168 × RB1190 → Spo ⁺

* Although previously described as *pyrA* (Buxton, 1976, 1980), from the origin and phenotype of the strain we now believe this to be *pyrX* (see Potvin *et al.*, 1975).

† *xhi-1479* is a prophage mutation of the defective phage PBSX causing heat-induction of this phage.

‡ *xki-1479* is a prophage mutation of PBSX causing the PBSX produced to have no killing activity on the sensitive strain W23.

§ *tal-1* is a mutation causing resistance to β-thienylalanine.

METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Media. Minimal agar was T-S agar based on Spizizen's salts (see Buxton, 1976). L-agar contained, per litre: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g; Difco agar, 16 g; adjusted to pH 7. Modified Schaeffer's medium (MSM) contained, per litre: Difco nutrient broth, 16 g; MgSO₄·7H₂O, 0.5 g; KCl, 2 g; adjusted to pH 7. This was freshly prepared, or re-steamed prior to use. Ca(NO₃)₂ (final concn 10⁻³ M), MnCl₂ (10⁻⁴ M), FeSO₄ (10⁻⁶ M) and glucose (0.1%, w/v) were added before use (Leighton & Doi, 1971). For MSM agar, 1.5% Difco agar was added.

Estimation of growth rates. Samples (0.6 ml) were added to 0.6 ml 10% formalin and the absorbance was measured at 600 nm using a Pye Unicam SP6-550 spectrophotometer. The *A*₆₀₀ was converted to bacterial dry wt ml⁻¹ by means of a standard calibration curve.

Selection of heat-resistant derivatives of a *Xhi*⁻ strain. Heat-resistant derivatives were selected for growth at 48 °C on Schaeffer's agar as described previously (Buxton, 1980).

Plate test for alkaline phosphatase. This was carried out as described previously (Piggot & Taylor, 1977) except that bacteria were grown on MSM agar.

Assay for alkaline phosphatase. This was performed 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.

Transformation. Transforming DNA was extracted as described by Marmur (1961). Competent bacteria were prepared according to Bott & Wilson (1968).

RESULTS

Isolation of deletion mutants that synthesize alkaline phosphatase constitutively

The *xhi-1479* mutation results in the heat-inducibility of prophage PBSX. Thus, a *xhi-1479* strain grows at 35 °C but lyses at 48 °C because of PBSX production (Buxton, 1976). Heat-resistant derivatives have been isolated which have deletions of all or part of the PBSX prophage together with some of the adjacent chromosome where the *pro(AB)* and *metC* loci are situated (Buxton, 1980). These deletion mutants, identified on the basis of their auxotrophy (Pro⁻ or Pro⁻Met⁻), were tested for formation of alkaline phosphatase during vegetative growth in the presence of inorganic phosphate, and all (three Pro⁻, and seven Pro⁻Met⁻) were found to be constitutive. This suggested that deletion of a locus mapping near *metC* (Piggot & Taylor, 1977) resulted in constitutive alkaline phosphatase synthesis. The locus was tentatively identified as *phoS* since this locus had been mapped near to *metC*, and *phoS* mutations caused constitutive enzyme synthesis (Piggot & Taylor, 1977).

In order to look for more such deletions, we constructed a *xhi-1479* strain (RB1180) in which the *spoIIA69* mutation was introduced by transformation. The *spoIIA* mutation prevented the phosphate-insensitive formation of alkaline phosphatase that occurs during spore formation, thus making it much easier to distinguish between strains constitutive and non-constitutive for enzyme formation during vegetative growth.

Ten separate cultures of RB1180 were grown at 32 °C in L-broth, and from each a single plate of MSM was inoculated with approximately 4×10^7 bacteria. These plates were incubated at 48 °C and gave an average of 85 colonies per plate. The colonies were replicated on to MSM, incubated at 48 °C, and also on to minimal agar, incubated at 35 °C. The master plates were then stained for alkaline phosphatase. In this way, putative phosphatase-constitutive colonies were identified. These were picked from the replica-plates and purified by single colony isolation on L-agar. By comparing the two types of replica-plates, auxotrophic mutants were also isolated.

Sixteen mutants constitutive for alkaline phosphatase were isolated in this way (approximately 2% of the heat-resistant colonies). They were derived from separate initial colonies and were therefore of independent origin. Three of the 16 mutants required proline and methionine for growth in addition to the uracil needed by the parental strain. These presumably had deletions from the PBSX prophage removing *pro(AB)* and *metC* (Buxton, 1980) [the methionine requirement was not satisfied by cystathionine or homocysteine; this is characteristic of a lesion in *metC* but not *metA* which also maps in the vicinity (see Garro *et al.*, 1970)]. No auxotrophic mutants were found that were not constitutive for alkaline phosphatase.

Taken together with the previously isolated deletion mutants (Buxton, 1980) selected on the basis of auxotrophy, four classes (numbered I to IV, Table 2) of heat-resistant derivatives could be distinguished. Type I could include, besides short deletions, some *xhi*⁺ revertants. For this reason, it is not possible to quantify the frequency of deletion formation, or the proportion of all deletions that had become alkaline phosphatase-constitutive.

The published three-point linkage data (Buxton, 1976, 1980) gives an order of markers in this region of: *argC-thiB-metA-xhi-pro(AB)-metC*. *phoS5* has been shown to be 37% cotransducible with *argC* and 77% cotransducible with *metC* (Piggot & Taylor, 1977), and this is compatible with a position for *phoS* between *xhi* and *pro(AB)* (see Fig. 1). The phenotypes of the deletion mutants support this conclusion, with mutants of classes I to IV representing progressively longer deletions extending from *xhi* through *phoS*, *pro(AB)* and *metC*.

Mapping of an alkaline phosphatase-constitutive deletion mutant with respect to phoS and sapA

To confirm that *phoS* was indeed deleted, genetic crosses were performed between strains having a point mutation in *phoS* and a strain with a short deletion (RB1190) (Table 3). The

Table 2. *Phenotypic classification of deletion mutants*

Heat-resistant derivatives of RB1037 (*xhi pyrX*) and RB1180 (*xhi pyrX rif spoIIA*) were selected at 48 °C. Deletion mutants were identified (a) from RB1037 as having an additional auxotrophic requirement besides Ura⁻, and (b) from RB1180 as producing alkaline phosphatase in the presence of phosphate.

Class	Growth on L-agar at 48 °C	Auxotrophic phenotype			Alkaline phosphatase constitutivity	Strain*
		Ura	Pro	Met		
Parent	—	—	+	+	—	RB1037 (a)
Parent	—	—	+	+	—	RB1180 (b)
I	+	—	+	+	—	RB1183 (a)
I	+	—	+	+	—	RB1184 (b)
II	+	—	+	+	+	RB1190 (b)
III	+	—	—	+	+	RB1081 (a)
IV	+	—	—	—	+	RB1084 (a)
IV	+	—	—	—	+	RB1193 (b)

* Representative strains only are given for heat-resistant mutants. The parental strain is indicated in parentheses — RB1037 as (a), RB1180 as (b).

Fig. 1. Linkage map derived from published linkage data, showing the extent of the deletions mentioned in the text. The numbers refer to percentage cotransduction using PBS1; arrowheads point in the direction of selected markers.

Table 3. *Transformation crosses between Δpho , and *phoS* and *sap* strains*

Donor	Colony phenotype	No. of transformant colonies*		
		SL351 (<i>sapA6 leuA8 spoIIE64</i>)	Recipient: SL345 (<i>phoS5 leuA8 spoIIE64</i>)	SL321 (<i>sapB2 leuA8 spoIIG55</i>)
SL340 (<i>phoS5</i>)	Leu ⁺	16 000	3420	1620
	Pho ⁻	7	0	15
RB1190 (Δpho)	Leu ⁺	25 700	4680	1110
	Pho ⁻	0	1	21
168 (<i>pho⁺ sap⁺</i>)	Leu ⁺	2000†	2900	500
	Pho ⁻	76	28	7

* For Leu⁺ transformants, the numbers counted have been corrected for dilution so as to give the estimated number of colonies on the plates from which the values for Pho⁻ colonies were obtained.

† Number not corrected for dilution; estimated on a crowded plate.

Table 4. Alkaline phosphatase activity in bacteria growing exponentially in Modified Schaeffer's medium at 37 °C

Strain	Relevant genotype	Alkaline phosphatase specific activity [units (mg dry wt) ⁻¹]
Heat-resistant derivatives of <i>xhi-1479</i> strains		
RB1190	$\Delta(\textit{phoS})$ <i>rif-2 spoIIA69</i>	19.6
SL1053	$\Delta(\textit{phoS-pro-metC})$ <i>rif-2 spoIIA69</i>	18.4
RB1084	$\Delta(\textit{phoS-pro-metC})$	8.8
RB1183	<i>pho</i> ⁺	0.6
Strains with point mutations in <i>phoS</i> and <i>sapA</i>		
SL340	<i>phoS5 rif-2 spoIIA69</i>	22.6
SL344	<i>phoS5</i>	8.4
SL349	<i>sapA6</i>	0.9

sapA locus also used in these crosses is close to, or identical to, *phoS* (Piggot & Taylor, 1977); *sapA* mutations affect the formation of alkaline phosphatase during sporulation, but not vegetative growth.

There is no direct selection for *phoS*⁺ (or *sap*⁺) and no suitable selective marker that is linked by transformation to *phoS*. This necessitated selection for an unlinked marker (*leu*⁺) using a saturating concentration of DNA; under such conditions up to about 5% of the prototrophic transformants would be expected to receive an unlinked marker, such as *pho*, as a result of a second transformation event (congression). Only this small proportion of the transformants can be regarded as 'useful' with respect to analysis of *pho*. The *pho*⁺ recombinants were detected as phenotypically Pho⁻ colonies amongst large numbers of Pho⁺ colonies. Donor DNA preparations were used at 10 µg ml⁻¹ which is saturating for these purposes, and all gave rise to reasonable congression frequencies when tested with a recipient, SL321, harbouring the unlinked *sapB2* mutation (Table 3). The recipient strains SL351 and SL345 gave 3.8% and 1% congression, respectively, of Pho⁻ with selection for *leu*⁺ when the parental strain 168 was used as donor. With RB1190 as donor, no Pho⁻ recombinants were obtained with SL351 (<0.004% of *leu*⁺) and only one colony with SL345 (<0.025% of *leu*⁺). With SL340 (*phoS5*) as donor and SL351, carrying the closely linked *sapA6* mutation, as recipient, seven Pho⁻ colonies (0.05% of *leu*⁺) were obtained.

The direct phosphatase staining procedure used killed the bacteria so that it was not possible to confirm the initial scoring by subculturing presumed Pho⁻ colonies (the alternative of replica-plating gave too many false negatives to be practicable). It is concluded that the deletion in RB1190 probably covers *phoS5* and *sapA6*, although it is possible that it ends short of, but very close to, these mutations.

Kinetics of alkaline phosphatase formation by the deletion mutants

All mutants deleted in the region of PBSX grew poorly in minimal media, and so enzyme formation was studied in bacteria growing exponentially in MSM. Under these conditions, *pho*⁺ strains (RB1183, Table 4, and RB1388, data not shown) produced little, or no, alkaline phosphatase. All the presumptive *pho* deletion mutants tested produced the enzyme constitutively. The differential plot of enzyme activity versus dry weight gave a linear relationship during exponential growth. The enzyme specific activity was very similar for the series of strains derived from RB1180 (RB1190 is shown) and also strain SL1053, a *spoIIA69* derivative of RB1084; this is consistent with the same gene being deleted in all strains.

Two alleles, *phoS5* and *phoS8*, were originally described for the *phoS* locus. *phoS5* gave a higher specific activity of alkaline phosphatase than *phoS8* (Piggot & Taylor, 1977). The

specific activity in strain SL340 (*spoIIA69 rif-2 phoS5*) was very similar to that in the deletion strains derived from RB1180 (e.g. RB1190); this is consistent with *phoS* being deleted in the latter.

All the above strains carried the mutations *spoIIA69 rif-2*. The *spoIIA69* mutation did not affect the constitutive level of alkaline phosphatase formation, as a *spo*⁺ transformant of RB1190, strain SL1059, had a very similar amount of enzyme to RB1190 (data not shown). However, the *rif-2* mutation (or an otherwise cryptic mutation closely linked to *rif*) did affect the constitutive rate, as two *Spo*⁺ *Rif*^s strains produced about half the amount of enzyme as strains differing only in the *spo* and *rif* loci (compare RB1084 with SL1053, and SL344 with SL340).

DISCUSSION

A series of heat-resistant, phosphatase-constitutive mutants have been derived from strain RB1180. These mutants are considered to be deletion mutants since the method used in their isolation is known to generate deletions (Buxton, 1980). Moreover, although selection was made only for heat-resistance, the mutants have, in some cases, also become auxotrophic for proline and methionine besides having lost the repression of phosphatase synthesis by inorganic phosphate. Indeed, constitutivity for phosphatase provides a rapid screening method for deletions generated from *Xhi*⁻ strains heat-inducible for PBSX.

The genes deleted almost certainly include *phoS* and *sapA*. The phosphatase phenotypes of all the deletions examined were the same as those of strains with the *phoS5* mutation but otherwise isogenic (Table 4). Thus, *phoS* is epistatic to *sapA* (if indeed *sapA* is a separate gene) with respect to formation of vegetative alkaline phosphatase in the presence of inorganic phosphate. The deletions provide no evidence for any other phosphatase gene in the region. As the deletions remove the *phoS* gene and lead to the same constitutive enzyme activity as a point mutation in *phoS*, it seems reasonable to conclude that the *phoS* gene product exerts negative control on alkaline phosphatase synthesis in the presence of inorganic phosphate. The constitutive level of synthesis was affected by mutation to rifampin-resistance, thus adding to the list of effects that such mutations can have (see Piggot *et al.*, 1981).

Strain RB1037 and its derivatives sporulated poorly, and, in any case, the constitutive vegetative production of alkaline phosphatase masks any effects during sporulation (Piggot & Taylor, 1977). Consequently, it was not possible to determine how the deletions affected the sporulation-associated production of alkaline phosphatase. There was, however, no obligatory requirement for any of the deleted region in forming a spore.

The method of deletion formation, involving the *xhi* mutation of PBSX, is of course only applicable to genes close to the PBSX prophage. A more generally applicable method of deletion formation is probably that involving the integration of a heat-inducible mutant of phage SP β (SP β c2) at secondary attachment sites and the subsequent isolation of heat-resistant derivatives (Rosenthal *et al.*, 1979; Zahler & Korman, 1981).

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