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Inorganic Phosphate Transport in *Escherichia coli*: Involvement of Two Genes Which Play a Role in Alkaline Phosphatase Regulation

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Two classes of alkaline phosphatase constitutive mutations which comprise the original *phoS* locus (genes *phoS* and *phoT*) on the *Escherichia coli* genome have been implicated in the regulation of alkaline phosphatase synthesis. When these mutations were introduced into a strain dependent on a single system, the *pst* system, for inorganic phosphate (P_i) transport, profound changes in P_i transport were observed. The *phoT* mutations led to a complete P_i^- phenotype in this background, and no activity of the *pst* system could be detected. The introduction of the *phoS* mutations changed the specificity of the *pst* system so that arsenate became growth inhibitory. Changes in the phosphate source led to changes in the levels of constitutive alkaline phosphatase synthesis found in *phoS* and *phoT* mutants. When glucose-6-phosphate or L-α-glycerophosphate was supplied as the sole source of phosphate, *phoT* mutants showed a 3- to 15-fold reduction in constitutive alkaline phosphatase synthesis when compared to the maximal levels found in limiting P_i media. However, these levels were still 100 times greater than the basal level of alkaline phosphatase synthesized in wild-type strains under these conditions. The *phoS* mutants showed only a two- to threefold reduction when grown with organic phosphate sources. The properties of the *phoT* mutants selected on the basis of constitutive alkaline phosphatase synthesis were similar in many respects to those of *pst* mutants selected for resistance to growth inhibition caused by arsenate. It is suggested that the *phoS* and *phoT* genes are primarily involved in P_i transport and, as a result of this function, play a role in the regulation of alkaline phosphatase synthesis.

Ever since the discovery that the synthesis of significant quantities of alkaline phosphatase (EC 3.1.3.1) in *Escherichia coli* occurred only under conditions of inorganic phosphate (P_i) limitation (10, 22), the specific role of P_i in repression of alkaline phosphatase synthesis has remained obscure. The current model for alkaline phosphatase regulation in *E. coli* (7) is based primarily on the study of constitutive mutants. These mutants, first isolated by Torriani and Rothman (23), produce significant amounts of alkaline phosphatase in media containing excess P_i or in media containing limiting P_i. Echols et al. (5) were able to map these constitutive mutations at two loci, *phoR* and *phoS*, distinct from the site of the alkaline phosphatase structural gene, *phoA*. Mutations at *phoR* and *phoS* were found to be recessive to the wild-type allele, indicating that cytoplasmic products might be involved.

Additional evidence suggests that the *phoR* product might also serve as an inducer of alkaline phosphatase synthesis. Most *phoR* constitutive mutants are unable to synthesize normal levels of alkaline phosphatase even under conditions of limiting P_i (8). Therefore, *phoR* mutants cannot be completely derepressed. Another class of mutants mapping at the *phoR* site, including several nonsense mutants, is completely alkaline phosphatase-negative (7); *cis-trans* tests indicate that these *phoR* mutants are deficient in a protein product required for the induction of alkaline phosphatase synthesis.

Constitutive mutations shown to lie in the

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phoS region can be divided into two classes. Approximately one-half of the mutants were found to be deficient in a protein, the R2A protein, which was purified and characterized by Garen and Otsuji (9). These authors also demonstrated that the synthesis of large amounts of the R2A protein occurred only under conditions of P_i limitation in the wild-type strain. In this communication, we will reserve the designation *phoS* for mutants lacking this protein (the original R2a$^-$ mutants of Garen and Otsuji [9]). The other group of constitutive mutants shown to be located in this region, which we will designate *phoT* (the original R2b$^-$ mutants of Garen and Otsuji [9]), synthesized normal amounts of the R2A protein. In addition to the evidence presented by Garen and Otsuji (9), we will provide a further functional basis for assigning these mutations to different cistrons (see below). Both *phoS* and *phoT* mutants synthesize alkaline phosphatase constitutively, and the latter also synthesize the R2A protein in the presence of excess P_i (9).

Using transductional analysis with phage P1, Aono and Otsuji (1) demonstrated that the two classes of mutations in the *phoS* region (in this communication, the *phoS* and *phoT* genes) can be mapped in the 73- to 74-min region on the *E. coli* chromosome (21) with the gene order *pyrE-tna-phoS(T)-ilv*. The locus *bgl*, which governs β-glucoside ulilization (19,20), is also located in this region of the *E. coli* chromosome. This is precisely the region of the chromosome where we have mapped (R. L. Bennett, Ph.D. thesis, Tufts Univ., Boston, Mass., 1972) the *pst* gene(s), mutations of which lead to alterations in a P_i transport system. Although the original *pst* mutants were isolated on the basis of resistance to arsenate (2) and were studied because of their altered P_i growth response, the implied role of P_i in alkaline phosphatase regulation led us to compare several of the properites of the original *phoS* (and *T*) mutants with those of the *pst* mutants.

In testing for the effects of *phoS* and *phoT* mutations on P_i transport, we have been careful to use strains especially constructed to be totally dependent on the *pst* transport system for P_i utilization (see Materials and Methods). It has been demonstrated that the ability to utilize P_i in *E. coli* is governed by at least four genetically distinct transport systems (2, 18; R. L. Bennett, Ph.D. thesis, Tufts Univ., 1972). All of the possible systems are not present in every *E. coli* strain examined.

The experiments reported here provide evidence that mutations of the *phoS* and *phoT* loci, although resulting in constitutive alkaline phosphatase synthesis, only indirectly affect the mechanism of alkaline phosphatase regulation. Rather, *phoS* and *phoT* are loci primarily involved with mechanisms of P_i transport and accumulation. The *phoT* cells lack a major P_i transport system, and the *phoS* cells are sensitive to arsenate, not normally a substrate for *pst*-mediated P_i transport (R. L. Bennett, Ph.D. thesis, Tufts Univ., 1972).

MATERIALS AND METHODS

Rationale. The existence of multiple systems for P_i transport in *E. coli* makes a study of the effects of mutations in one of the systems impossible unless the other systems are removed or controlled.

To test for the effects of *pst*, *phoS*, and *phoT* mutations on P_i transport, it was necessary to construct a strain dependent on the *pst* system for all P_i transport. This required the elimination of the inducible glycerol phosphate transport system (*glpT*; 16) and the inducible glucose-6-phosphate transport system (*uhp*; 12), both of which are capable of transporting P_i as a secondary substrate (Bennett and Malamy, *in preparation*). Strains UR1011 and GR5154 (see below) were made *glpT*$^-$ by conjugation. Since glucose-6-phosphate was not used in the experiments of this type, the glucose-6-phosphate transport system was never induced.

Another constitutive phosphate transport system which we have designated *pit* (P_i transport) can also transport P_i and arsenate into the cell. However, the *pit* system is not present in all strains tested; strains derived from 13.6 (see below) are *pit*$^+$, whereas strains derived from K10 and U7 do not contain a functional *pit* system (Willsky, Bennett, and Malamy, *in preparation*). Strains UR1011 and GR5154 were derived to contain the corresponding *pit* locus from U7. In minimal media with P_i as the sole source of phosphorus, UR1011 and GR5154 are completely dependent on the *pst* system for growth; elimination of the *pst* system by mutation results in a strain unable to utilize P_i (2; R. L. Bennett, Ph.D. thesis, Tufts Univ., 1972).

Bacterial strains. All strains used in this study are listed in Table 1. UR1, UR3, and UR13 were isolated as arsenate-resistant (Asi^r) mutants of U7 (2). Strain UR1011 was constructed to contain only one functional phosphate transport system by transducing UR1, one of the completely phosphate-negative strains (P_i^-), to P_i^+ with phage P1 grown on a P_i^+, *ilv*$^-$ host (AB2277). The P_i^+, *ilv*$^-$ recombinant selected has received the *pst*$^+$ gene(s) located at min 74, which specifies the only phosphate transport system in this strain. Strain GR5154 contains the segment of the chromosome from *ilv* to *mtl* derived from UR1011 and in addition is *phoA*$^+$, *glpT*$^-$, and *bgl*$^+$.

Chemicals. Salicin was purchased from Schwartz-Mann, *p*-nitro-phenylphosphate (Sigma 104), glucose-6-phosphate, and DL-α-glycerophosphate were obtained from Sigma Chemical Co., and radioactive

TABLE 1. *Bacterial strains*

Strain	Genotype[a]	Source
	Strains from this laboratory	
U7	Hfr Cavalli, *phoA*$^-$, *bgl*$^-$, *pst*$^+$, *glpT*$^+$	A. M. Torriani (2)
UR1	Hfr Cavalli, *phoA*$^-$, *bgl*$^-$, *pst*$_{UR1}$$^-$, *glpT*$^-$	Asi^r mutants of U7 (2)
UR3	Hfr Cavalli, *phoA*$^-$, *bgl*$^-$, *pst*$_{UR3}$$^-$, *glpT*$^-$	Asi^r mutants of U7 (2)
UR13	Hfr Cavalli, *phoA*$^-$, *bgl*$^-$, *pst*$^+$, *glpT*$^-$	Asi^r mutants of U7 (2)
UR1011	Hfr Cavalli, *phoA*$^-$, *bgl*$^-$, *pst*$^+$, *glpT*$^-$, *ilv*$^-$	P_i^+ transductant of UR1 (P1·AB2277)
GR5134	F$^-$, *phoA*$^+$, *bgl*$^+$, *pst*$^+$, *glpT*$^-$	This study[c]
GR5154	F$^-$, *phoA*$^+$, *bgl*$^+$, *pst*$^+$, *glpT*$^-$, *ilv*$^-$	This study[d]
	Alkaline phosphatase constitutive strains obtained from other laboratories	
C10	Hfr Reeves 1, *phoA*$^+$, *bgl*$^-$, (*pst*$^+$), *phoS*$^+$, *phoT*$_{C10}$	A. M. Torriani (5)
C90	Hfr Cavalli, *phoA*$^+$, *bgl*$^-$, (*pst*)$^+$, *phoS*$^+$, *phoT*$_{C90}$	A. Garen (9)
C101	F^{-b}, *phoA*$^+$, *bgl*$^-$, (*pst*)$^+$, *phoS*$^+$, *phoT*$_{C101}$$^-$	A. Garen (9)
C112	F^{-b}, *phoA*$^+$, *bgl*$^-$, (*pst*)$^+$, *phoS*$^+$, *phoT*$_{C112}$$^-$	A. Garen (9)
C72	Hfr Cavalli, *phoA*$^+$, *bgl*$^-$, *pst*$^+$, *phoS*$_{C72}$$^-$, *phoT*$^+$	A. Garen (9)
C78	Hfr Cavalli, *phoA*$^+$, *bgl*$^-$, *pst*$^+$, *phoS*$_{C78}$$^-$, *phoT*$^+$	A. Garen (9)
C86	F^{-b}, *phoA*$^+$, *bgl*$^-$, *pst*$^+$, *phoS*$_{C86}$$^-$, *phoT*$^+$	A. Garen (9)
	Other strains	
K10	Hfr Cavalli, *phoA*$^+$, *bgl*$^-$, *pst*$^+$, *glpT*$^+$, *phoS*$^+$, *phoT*$^+$	A. M. Torriani
AB2277	F$^-$, *phoA*$^+$, *pst*$^+$, *ilv*$^-$	E. Adelberg
13.6	F$^-$, *phoA*$^+$, *bgl*$^-$, *pst*$^+$, *glpT*$^+$, *ilv*$^+$	J. Davies
XS1	HfrH, *phoA*$^+$, *bgl*$^-$, *pst*$^+$, *glpT*$^+$, *ilv*$^+$	W. Epstein

[a] The conventions of Demerec et al. (4) and the nomenclature incorporated in the latest *E. coli* map (19) have been used to describe cell genotypes. Only those markers relevant to this study are indicated. The marker *glpT* codes for a L-α-glycerophosphate permease (16) which utilizes P_i as a secondary substrate, *phoA* codes for the structural gene for alkaline phosphatase (22), *bgl* governs β-glucoside utilization (19, 20), and *ilv* is concerned with the formation of isoleucine and valine (21). The following new designations have been adopted: *pst*, phosphate specific transport (Bennett, Willsky, and Malamy, *unpublished data;* R. L. Bennett, Ph.D. thesis, Tufts Univ., 1972); *phoS* alkaline phosphatase constitutive mutants lacking the R2A protein (the original R2a$^-$ mutant of Garen and Otsuji); *phoT*, alkaline phosphatase constitutive mutants retaining the R2A protein (includes several of the *phoS* mutants originally designated R2b$^-$).

[b] Although these strains had been isolated from K10, an Hfr Cavalli, no vestige of the sex factor could be detected in these strains.

[c] GR5134 was constructed from 13.6 by a series of manipulations which included: (i) introduction of *phoA*$^-$ from U7, (ii) introduction of *glpT*$^-$ from UR13, (iii) introduction of *phoA*$^+$ from XS1, and (iv) selection of a spontaneous *bgl*$^+$ colony on MacConkey salicin plates.

[d] GR5154 is an exconjugant of UR1011 and GR5134 selected to contain the region of the chromosome from *ilv* to *mtl* from UR1011. Since the exconjugant was *bgl*$^-$, a spontaneous *bgl*$^+$ colony was selected on MacConkey salicin plates.

orthophosphate ($^{32}P_i$) was obtained from New England Nuclear Corp.

Enriched media. LC broth was L broth (13) without the glucose and supplemented with 2 mM $CaCl_2$. L plates were prepared by adding 1.5% agar to L broth. LM (14) plates were used as enriched limiting phosphate medium. Indicator plates to score the *bgl* marker (19, 20) were prepared from Difco MacConkey agar base with the addition of 10 g of salicin per liter.

Minimal media. The basic minimal medium was WT (2) supplemented with 2 mg of thiamine per liter. Sterile carbon and inorganic or organic phosphate sources were added separately to the autoclaved WT base. Solid media were prepared by adding 0.1% sodium citrate and 1.5% agar. Amino acids when required were added at 5 mg/liter.

Media and methods for scoring phosphate and arsenate response. The basic WT liquid or solid medium with appropriate additions to make complete media was used for scoring parental strains and P1 transductants. The following complete solid media were used: Gly·GlyP (0.6% glycerol, 5×10^{-5} M P_i, 5×10^{-4} M DL-α-glycerophosphate); Gly·P_i (0.6% glycerol, 10^{-3} M P_i); Gly·P_i·Asi (0.6% glycerol, 5×10^{-4} M P_i, 5×10^{-3} M arsenate); Glu·P_i (0.6% glucose, 10^{-3} M P_i); Glu·P_i·Asi (0.6% glucose, 5×10^{-4} M P_i, 5×10^{-3} M arsenate). For liquid media, the concentrations of arsenate and P_i in Glu·P_i·Asi and Gly·P_i·Asi media were 10 times higher.

P_i^+ strains are characterized by normal growth on Glu·P_i, Gly·P_i, and Gly·GlyP media. P_i^- strains are unable to grow on Gly·P_i or Glu·P_i media, but growth on Gly·GlyP remains normal. Arsenate sensi-

tivity (Asi^s) is defined as the inability of a strain to grow on Glu·P_i·Asi and Gly·P_i·Asi while growing normally on Glu·P_i and Gly·P_i.

Transductants were purified once on the same medium used for selection (Gly·GlyP + amino acids) and then were scored for phosphate and arsenate growth response by replica-plating onto the appropriate media. Since the growth response on solid media was sometimes ambiguous, transductants were also scored in liquid media.

P1 transduction. Stocks of P1kc (obtained from D. Morse) for transduction were prepared according to a protocol suggested by D. Morse. All strains to be used for P1 production were grown to mid-exponential phase in LC broth. Equal volumes of bacteria and various P1 dilutions in LC broth were mixed to give a final volume of less than 0.5 ml and were incubated at 37 C for 20 min. A 3-ml portion of LC soft agar (LC + 0.6% agar) was added to the mixture, which was then poured onto fresh (less than 48 hr old) LC plates. After overnight incubation at 37 C, the plate showing almost confluent growth of plaques was flooded with 5 ml of LC broth and left at room temperature for 3 hr. The solution was removed without distrubing the soft agar layer, centrifuged to remove cell debris, and stored at 4 C with a few drops of chloroform. This procedure was repeated at least twice before a stock was used for transduction.

For transduction, the recipient, grown to late-exponential phase in LC broth, was mixed with suitable dilutions of P1 to give a multiplicity of infection of 0.5. After incubation at 37 C for 20 min, the cells were centrifuged at room temperature, washed and resuspended in SSC (0.15 M NaCl, 0.15 M sodium citrate), and plated on selective media.

Plate assay for alkaline phosphatase. Cells were grown at 37 C on L plates (for excess phosphate medium) or LM plates (for limiting phosphate medium). A circle of Whatman no. 1 filter paper soaked in a solution of 0.5 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.0, containing 20 mg of *p*-nitrophenyl phosphate (PNPP) per ml was placed directly onto the colonies or patches. An intense yellow color developed over the alkaline phosphatase-containing colonies within 5 min at room temperature. All strains genetically alkaline phosphatase-positive give rise to yellow colonies on LM plates, whereas only alkaline phosphatase constitutive colonies produce yellow color on L plates.

Quantitative spectrophotometric assay for alkaline phosphatase. A 5-ml portion of cell culture was centrifuged and then was washed and resuspended in the same volume of 0.5 M Tris buffer, *p*H 8.0. The cell suspensions were treated with toluene (0.3 ml) and shaken at 37 C for 30 min. Assays were performed at room temperature in a final volume of 1.0 ml of 0.5 M Tris buffer, *p*H 8.0, containing 0.2 mg of PNPP per ml and samples of the cell suspension. Liberation of *p*-nitrophenol, measured by changes in absorbance at 420 nm, was followed in a Gilford recording spectrophotometer. Units of alkaline phosphatase specific activity, as used throughout this paper, are defined as the change in absorbance at 420 nm per minute per unit of optical density at 600 nm (cell turbidity) of the cell suspension.

Phosphate transport assays. $^{32}P_i$ was purchased as the sodium salt in dilute HCl. A working solution of $^{32}P_i$ diluted with sodium phosphate to the desired specific activity was prepared at least 24 hr before an experiment and was filtered three times through membrane filters (Millipore Corp.; type HA, 0.45 μm pore size) just prior to use (17). P_i concentrations were determined by the method of Berunblum and Chain (3).

Cells were grown to mid-log phase in WT media containing 0.6% glucose and 10^{-3} M P_i. The cells were maintained at constant cell density by dilution with prewarmed medium (37 C). A 10-ml sample was filtered on a membrane filter, washed with 10 ml of prewarmed WT medium, and resuspended in WT medium containing 100 μg of chloramphenicol/ml and 0.6% glucose. This suspension was incubated for 3 min at 37 C with aeration before $^{32}P_i$ was added. Samples (1 ml) were then removed at suitable intervals, and the cells were collected on a membrane filter and washed with chilled WT containing 10^{-3} M P_i. To secure the filters to the planchets, the filters were placed in aluminum planchets, dissolved with 1 ml of acetone, and then dried under a heat lamp. Radioactivity was determined in a Nuclear-Chicago gas-flow counter. For K_m determinations, the initial velocity is calculated for the interval between 0.2 and 1.0 min.

RESULTS

P_i transport in alkaline phosphatase constitutive cells. Although the growth of alkaline phosphatase constitutive cells appeared normal in media containing 0.01 M P_i as the sole source of phosphorus, a kinetic analysis of P_i uptake in these strains revealed a striking difference. The *phoS* strains, of which C86 serves as an example in Table 2, had similar K_m and V_{max} values for P_i uptake when compared with strains K10 and U7, taken as the wild type for P_i transport. However, the *phoT* strains C90 (Table 2) and C112 (data not shown) showed a 100-fold reduction in affinity for P_i. This reduction in affinity was also revealed as a requirement for higher concentrations of P_i in order for *phoT* strains to attain normal growth rates in minimal media. The generation time of *phoT* mutant C90 growing in WT medium increased as the P_i concentration was lowered. In medium containing glucose and 10^{-4} M P_i the generation time of C90 was three times longer than the generation time observed in medium containing glucose and 10^{-2} M P_i. The wild-type strain U7 and the *phoS* mutants C78 and C86 attained the same generation time at either P_i concentration.

Transfer of phoS, phoT and pst mutations into UR1011. To test for the effect of

TABLE 2. *Characterization of P_i transport in strain K10 and its derivatives*

Strain	Genotype	K_m for P_i (μM)	V_{max}[a]
K10	*phoA*$^+$, *pst*$^+$, *phoS*$^+$, *phoT*$^+$	5.5	7.7
U7	*phoA*$^-$, *pst*$^+$, *phoS*$^+$, *phoT*$^+$	3.2	8.2
C86	*phoA*$^+$, *pst*$^+$, *phoS*$_{C86}^-$, *phoT*$^+$	1.5	2.4
C90	*phoA*$^+$,(*pst*)$^+$, *phoS*$^+$, *phoT*$_{C90}^-$	383	8.1

[a] Expressed as nanomoles per minute per unit of optical density at 600 nm.

phoS and *phoT* mutations on P_i transport mediated by the *pst* gene(s), it was necessary to introduce these mutations into a strain totally dependent on the *pst* system for P_i transport. UR1011, a P_i^+ transductant of UR1, is capable of normal growth on liquid and solid minimal media containing 10^{-3} M P_i, and in addition is as resistant to inhibition by arsenate as is UR1 (2). When UR1011 was transduced to *ilv*$^+$ with P1 grown on two known *pst* mutants, UR1 and UR3 (R. L. Bennett, Ph.D. thesis, Tufts Univ., 1972), the P_i^- phenotype was introduced into 32 to 35% of the selected recombinants (Table 3). Similarly, when P1 grown on the *phoT* mutants C10, C90, C101, and C112 was employed, between 25 and 57% of the *ilv*$^+$ transductants became P_i^-. By contrast, *ilv*$^+$ transductants derived from crosses with P1 grown on *phoS* mutants C78 and C86 always remained P_i^+. However, when these *ilv*$^+$ recombinants were tested on plates containing both P_i and arsenate (Gly·P_i·Asi and Glu·P_i·Asi), 33 to 47% had become Asi^s. Thus, among *ilv*$^+$ recombinants of UR1011, the inability to utilize P_i can be introduced from *phoT* and *pst* mutants with approximately the same frequency as the introduction of Asi^s from *phoS* mutants.

Transfer of phoS, phoT and pst mutations into strain GR5154. To examine the relationship between alkaline phosphatase constitutivity and the Asi^s observed with transductants derived from *phoS* mutants, a *phoA*$^+$ derivative was required. Strain GR5154, which is phenotypically similar to strain UR1011 for all known P_i transport systems but in addition is *phoA*$^+$ and *bgl*$^+$, was employed. Table 4 demonstrates that, among the *ilv*$^+$ transductants of GR5154 obtained with P1 grown on three different *phoS* donors, alkaline phosphatase constitutivity and Asi sensitivity were 100% linked. This strongly suggests that these properties are determined by the same gene.

The *bgl*$^+$ marker in GR5154 allows a comparison of the linkage of *phoS* and *phoT* mutations with that of *pst* mutations with respect to another marker (*bgl*$^+$) in this region of the chromosome. The cotransduction of *phoS*, *phoT*, and *pst* with *bgl* occurred with approximately the same frequencies in all cases (Table 4).

Quantitative assays of alkaline phosphatase activity revealed that constitutive alkaline phosphatase synthesis in GR5154 transductants containing *phoS* and *phoT* mutations is almost 10 times higher than that observed with transductants containing the *pst* mutations UR1 and UR3 in medium containing 0.6% glycerol and 5×10^{-4} M DL-α-glycerophosphate (Table 5). This result suggests that *phoT* and *pst* mutants might represent different genes, even though both types of mutants can serve to introduce the P_i^- phenotype into suitable recipients.

Effect of different phosphate sources on constitutive alkaline phosphatase synthesis. Having shown an involvement of the *phoS* and *phoT* genetic loci in P_i transport,

TABLE 3. *Co-transduction frequencies of phoS, phoT, and pst with ilv*$^+$ *in strain UR1011*

Donor strain	No. of *ilv*$^+$ recombinants scored	Phosphate and arsenate phenotype[a]	Percentage of total *ilv*$^+$
phoS mutants			
C78	120	P_i^+, Asi^r	53
		P_i^+, Asi^s	47
C86	120	P_i^+, Asi^r	67
		P_i^+, Asi^s	33
phoT mutants			
C10[b]	80	P_i^+	43
		P_i^-	57
C90	61	P_i^+	58
		P_i^-	42
C101	120	P_i^+	75
		P_i^-	25
C112	120	P_i^+	68
		P_i^-	32
pst mutants			
UR1[b]	120	P_i^+	68
		P_i^-	32
UR3[b]	120	P_i^+	65
		P_i^-	35

[a] Phosphate and arsenate phenotypes were determined by replica-plating onto selective medium as described in Materials and Methods.

[b] The preparation of P1 for these experiments was modified from the procedure in Materials and Methods. P1 was plated on LC plates as before but after a 4- to 6-hr incubation at 37 C the plate showing confluent lysis was flooded with 5 ml of LC broth and left overnight at 4 C before harvesting.

TABLE 4. *Co-transduction frequencies of phoS, phoT, pst with ilv⁺ in strain GR5154*

Donor strain	No. of *ilv*⁺ recombinants scored	Classes found[a]	Percentage of total *ilv*⁺
phoS mutants			
C72	181[b]	*bgl*⁺, *phoS*⁺, P_i^+, Asi^r	81.2
		bgl⁻, *phoS*⁻, P_i^+, Asi^s	12.1
		bgl⁻, *phoS*⁺, P_i^+, Asi^r	6.7
C78	112[c]	*bgl*⁺, *phoS*⁺, P_i^+, Asi^r	72.3
		bgl⁻, *phoS*⁻, P_i^+, Asi^s	25.9
		bgl⁻, *phoS*⁺, P_i^+, Asi^r	1.8
C86	176[b]	*bgl*⁺, *phoS*⁺, P_i^+, Asi^r	73.3
		bgl⁻, *phoS*⁻, P_i^+, Asi^s	23.9
		bgl⁻, *phoS*⁺, P_i^+, Asi^r	2.8
phoT mutant[d]			
C90	174[b]	*bgl*⁺, *phoT*⁺, P_i^+	85.0
		bgl⁻, *phoT*⁻, P_i^-	12.7
		bgl⁻, *phoT*⁺, P_i^+	2.3
pst mutants			
UR1	195[b]	*bgl*⁺, *pst*⁺	71.3
		bgl⁻, *pst*⁻	26.1
		bgl⁻, *pst*⁺	2.6
UR3	30	*bgl*⁺, *pst*⁺	70.0
		bgl⁻, *pst*⁻	30.0

[a] Scoring of *bgl*⁺ was done on MacConkey salicin plates; alkaline phosphatase constitutivity (*phoS*⁻ or *phoT*⁻) was scored by the alkaline phosphatase plate assay; phosphate and arsenate responses were scored by replica-plating.

[b] Two hundred *ilv*⁺ transductants were picked for purification, but some of them were not stable and could not be scored.

[c] One hundred twenty *ilv*⁺ transductants were originally picked for purification.

[d] This transduction was also undertaken with the *phoT* mutants C101 and C112 as donors, but a greatly reduced *bgl-ilv* linkage was observed. The reason for this lowered linkage is not known at this time.

TABLE 5. *Constitutive alkaline phosphatase synthesis in GR5154 transductants carrying phoS, phoT, and pst mutations*

Strain	Phosphate and arsenate phenotype[a]	Origin of *ilv-bgl* region	Alkaline phosphatase specific activity[b]
GR5154	P_i^+ Asi^r	Parental	<0.02
GR5156	P_i^+ Asi^s	C86 (*phoS*⁻)	4.0
GR5158	P_i^+ Asi^s	C72 (*phoS*⁻)	3.9
GR5160	P_i^+ Asi^s	C78 (*phoS*⁻)	4.3
GR5157	P_i^-	C90 (*phoT*⁻)	3.6
GR5159	P_i^-	UR3 (*pst*⁻)	0.47
GR5161	P_i^-	UR1 (*pst*⁻)	0.51

[a] Phosphate and arsenate phenotypes were determined by replica-plating.

[b] Expressed as change in optical density at 420 nm per minute per unit of optical density at 600 nm. Cells were grown to stationary phase in media containing 0.6% glycerol and 5×10^{-4} M DL-α-glycerophosphate. Alkaline phosphatase activity was measured spectrophotometrically.

we then investigated the possibility that the constitutive production of alkaline phosphatase in these strains was due solely to limiting amounts of internal P_i. According to the above hypothesis, if the internal P_i concentration could be elevated by growth on different phosphate sources, then one might see a reduction in the constitutive level of alkaline phosphatase synthesis. Since *phoT* mutants in our genetically defined background (GR5154) are unable to utilize P_i (as a phosphate source) or DL-α-glycerophosphate (as a carbon and phosphate source), it was necessary to use the constitutive mutants as originally isolated (5) for the series of experiments requiring growth in media containing P_i or DL-α-glycerophosphate.

It should be emphasized that the original *phoT* mutants C90, C112, and C101 which we obtained from the laboratory of A. Garen, as derivatives of strain K10, are P_i^+ and seem to have another system for P_i transport which can supply adequate P_i for growth. This system may also be present in the *phoS* mutants obtained from Garen's laboratory. Although we have not encountered this system in our derivatives of K10 (U7, UR1, UR3, UR1011), there is another system, designated *pit* (P_i transport), which is known to be present in several of our strains derived from strain 13.6. Strain GR5154, originally in this series, is also lacking this system since it now contains the inactive *pit* region from UR1011 (Bennett, Willsky, and Malamy, *unpublished data;* R. L. Bennett, Ph.D. thesis, Tufts Univ., 1972).

In agreement with the early studies of Torriani (22), we found that alkaline phosphatase synthesis is completely repressed in the wild-type K10 at P_i concentrations in the medium in excess of 6×10^{-4} M (Fig. 1A). At P_i concentrations below this value, alkaline phosphatase synthesis is derepressed, and at 10^{-4} M P_i it reaches a level 400- to 500-fold greater than the repressed basal level at 10^{-3} M, with either glycerol or glucose as the carbon source (Fig. 1A and Table 6).

The *phoS* mutant C86 synthesized at least twice as much alkaline phosphatase as the fully derepressed wild-type strain even at 10^{-3} M P_i (Fig. 1B). Further reduction in medium P_i concentration resulted in only a slight increase in alkaline phosphatase level. In the experiment reported here, C86 cells grown in glycerol media contained about 30 to 40% more alkaline phosphatase activity than glucose-grown cells at all P_i concentrations employed (Fig. 1B and Table 6).

TABLE 6. *Alkaline phosphatase synthesis in K10 and constitutive derivatives grown under various conditions*

Growth conditions	Alkaline phosphatase specific activity[a]						
	Wild-type K10	*phoS* mutants			*phoT* mutants		
		C72	C78	C86	C90	C101	C112
Glucose-6-phosphate, 0.03 M[b]	<0.01	3.3	3.5	4.5	3.3	1.6	5.9
Glucose, 0.6%; P_i, 10^{-2} M[b]	<0.01	3.5	3.2	6.2	2.8	1.4	7.3
Glucose, 0.6%; P_i, 10^{-4} M[b]	3.6	9.4	8.9	10.3	10.4	5.7	8.4
DL-α-Glycerophosphate, 0.03 M[c]	<0.01	4.6	5.0	3.5	0.7	1.8	1.9
Glycerol, 0.6%; P_i, 10^{-2} M[c]	<0.01	4.1	4.1	3.8	3.3	2.2	3.3
Glycerol, 0.6%; P_i, 10^{-4} M[c]	4.6	11.1	11.2	9.7	11.0	8.3	9.0

[a] Expressed as change in optical density at 420 nm per minute per unit of optical density at 600 nm.

[b] Cells were grown to stationary phase (at 37 C) in WT medium containing 0.03 M glucose-6-phosphate as the organic phosphate source. The cells were harvested by centrifugation, washed once with WT, and diluted into WT medium with the indicated additions. These cultures were grown to stationary phase at 37 C and then assayed for alkaline phosphatase spectrophotometrically.

[c] The cells were treated as described in the preceding footnote except that DL-α-glycerophosphate was the organic phosphate source used.

The *phoT* mutant C90 exhibited an entirely different response to changes in medium P_i concentrations. Although alkaline phosphatase synthesis in C90 at 10^{-3} M P_i approached the fully derepressed wild-type level, further reduction in medium P_i to 3×10^{-4} M led to a twofold increase in alkaline phosphatase content for glucose-grown cells; at 10^{-4} M P_i, alkaline phosphatase levels for C90 grown in glycerol approached the higher level typical of that found in *phoS* mutants grown under the same conditions. Glucose-grown cells of C90 reached the highest alkaline phosphatase levels measured in these experiments (Fig. 1C) at 10^{-4} M P_i.

Growth of *phoS* and *phoT* mutants on glucose-6-phosphate as the sole source of carbon and phosphorus resulted in levels of constitutive alkaline phosphatase synthesis similar to those found after growth in glucose and excess P_i (10^{-2} M; Table 6). Growth of *phoS* mutants on DL-α-glycerophosphate as the sole source of carbon and phosphorus resulted in levels of constitutive alkaline phosphatase synthesis similar to those found after growth in glycerol and excess P_i (10^{-2} M). However, *phoT* mutants grown in DL-α-glycerophosphate as the sole source of carbon and phosphorus had a lower constitutive alkaline phosphatase level than *phoS* mutants in the same medium. Transfer of the DL-α-glycerophosphate-grown cells to glycerol media with excess P_i (10^{-2} M) led to a small increase in alkaline phosphatase levels for mutants C101 and C112 and to a sevenfold increase for mutant C90. Transfer to limiting P_i medium (10^{-4} M) with glycerol as the carbon source led to even greater levels of alkaline phosphatase. In the case of mutant C90, there was a 16-fold difference in the level of alkaline phosphatase between cells grown with DL-α-glycerophosphate and cells grown with glycerol and limiting P_i. The other two mutants showed a fivefold difference under the same conditions. When the *phoT* mutant C90 was grown in DL-α-glycerophosphate, the constitutive levels of alkaline phosphatase synthesis were greatly reduced in comparison to the same cells grown in glycerol and limiting P_i.

The wild-type K10 failed to synthesize alkaline phosphatase at greater than the basal level with either organic phosphate source or when grown with excess P_i (Table 6). A 400- to 500-fold increase in alkaline phosphatase activity was found in cells of K10 grown with limiting P_i and glucose or glycerol.

From the results reported in Table 6, it is possible to conclude that growth on different sources of phosphorus can affect the level of alkaline phosphatase production in constitutive mutants; the change is greater for *phoT* mutants than for *phoS* mutants.

Kinetics of "derepression" for alkaline phosphatase constitutive strains grown with organic phosphates. Our results show that the highest level of alkaline phosphatase synthesis occurred after growth of the mutants in the low P_i media, conditions leading to maximal alkaline phosphate synthesis in the wild-type strain. In addition, a reduction in constitutive alkaline phosphatase synthesis occurred after growth of the constitutive mutants in media containing organic phosphates or high P_i as the phosphate source. However, in these experiments none of the phosphate sources tested led

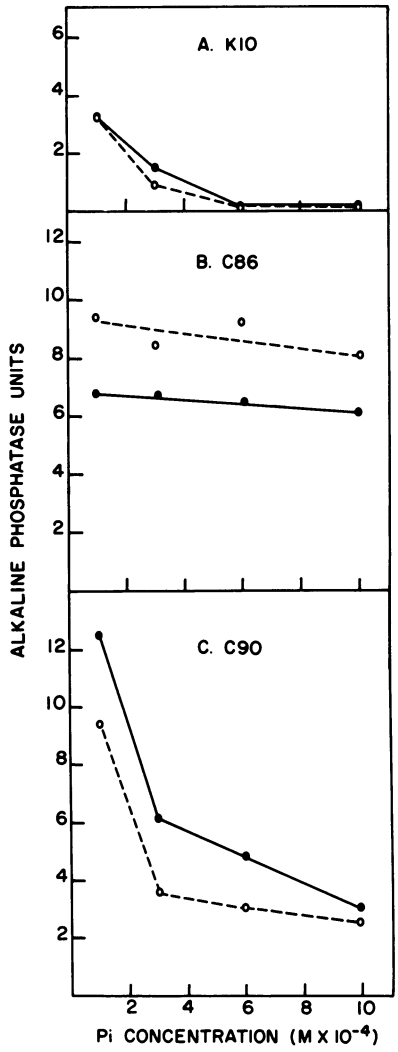

FIG. 1. *Constitutive alkaline phosphatase synthesis as a function of inorganic phosphate concentration. Cells were grown at 37 C to stationary phase in WT media containing 0.03 M L-α-glycerophosphate or glucose-6-phosphate. The cells were harvested by centrifugation, washed in WT, and resuspended as follows: cells grown with glycerophosphate were resuspended in 0.6% glycerol and the indicated inorganic phosphate concentration (○); cells grown with glucose-6-phosphate were resuspended in WT with 0.6% glucose and the indicated inorganic phosphate concentration (●). Incubation was continued at 37 C until stationary phase, and alkaline phosphatase activity was determined spectrophotometrically as described in Materials and Methods.*

to the reduction of alkaline phosphatase synthesis to the wild-type repressed level. The difference in alkaline phosphatase levels reported in Table 6 for cells grown in media containing high P_i or organic phosphate compared with cells grown in media containing low P_i seems to result from derepression of alkaline phosphatase synthesis in these constitutive mutants in response to decreasing P_i concentration. It should be possible to document this rise in alkaline phosphatase synthesis when the cells are transferred from media containing organic phosphate to media containing low P_i.

When cultures of *phoS* and *phoT* mutants were grown with glucose-6-phosphate as the sole source of carbon and phosphorus and then transferred to media with glucose and excess (10^{-2} M) or limiting (10^{-4} M) P_i, the phenomenon of further alkaline phosphatase derepression was clearly seen. The *phoT* mutant C90 showed a large increase in alkaline phosphatase content for cells grown with limiting P_i when compared to the same cells grown with glucose-6-phosphate or excess P_i. A similar, although not as marked, effect was seen with the *phoS* mutant C86. In both cases, the constitutive level of alkaline phosphatase synthesis was increased from that found in the cells grown with glucose-6-phosphate by transfer of the cells to media with limiting P_i (Fig. 2A and B).

The derepression effect was even more marked when *phoS* and *phoT* mutants were grown with glycerophosphate and then transferred to media with glycerol and excess or limiting P_i. C90 showed a constant level of alkaline phosphatase activity during growth in glycerophosphate or excess P_i (Fig. 2C), and a large increase in alkaline phosphatase content when transferred to limiting P_i. This result is in accord with the values reported for C90 in Table 6. A large increase in alkaline phosphatase activity was also seen when C86 was transferred to limiting P_i (Fig. 2D). The experiments reported in Fig. 2 show that the level of alkaline phosphatase activity found in *phoS* and *phoT* cells grown with organic phosphates can be increased by transfer of the cells to media with limiting P_i.

DISCUSSION

The current model for the control of alkaline phosphatase synthesis in *E. coli* (7) defines the role of the R1 protein, product of the *phoR* gene, as that of an inducer, the presence of which is required for the transcription of the *phoA* gene. The role of the *phoS* product (the R2A protein), and the protein specified by the *phoT* gene, in repression of alkaline phosphatase synthesis remains unclear. The most prominent small molecule playing a part in alkaline phosphatase regulation is P_i. It has

been suggested (8) that intracellular P_i serves as a co-repressor or co-factor in a series of reactions involving the *phoS* and *phoT* products which lead to the inactivation of the R1 protein. Most of the evidence supporting this scheme comes from the study of constitutive mutants at the *phoR*, *phoS*, and *phoT* loci.

In this paper, we have shown that mutations affecting P_i transport, which have been mapped at the *pst* locus located between 73 and 74 min on the *E. coli* chromosome, result in constitutive alkaline phosphatase synthesis. We have also shown that *phoS* and *phoT* constitutive mutations, when transferred to a suitable genetic background (GR5154), lead to changes in P_i transport and accumulation. These observations suggest that there might be a pathway of P_i transport and metabolism

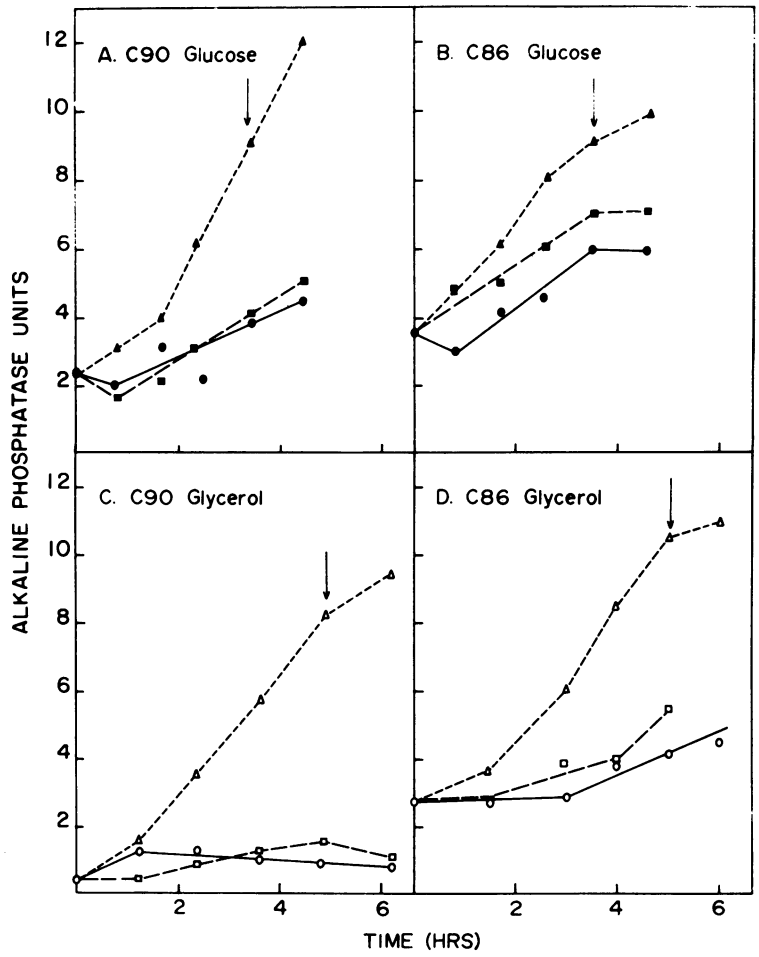

FIG. 2. *Derepression of alkaline phosphatase synthesis in phoS and phoT mutants as a function of time. (A and B). Cells were grown to stationary phase at 37 C in WT medium containing 0.03 M glucose-6-phosphate as the organic phosphate source. The cells were harvested by centrifugation, washed once with WT, and diluted into fresh medium. After 3 hr of further incubation at 37 C, a sample was removed for the zero-time alkaline phosphatase assay, the culture divided into thirds and harvested and washed as before. The cells were then resuspended in WT media with the following additions: 0.03 M glucose-6-phosphate (●), 0.6% glucose and 10^{-2} M P_i (■), 0.6% glucose and 10^{-4} M P_i (▲). Samples were removed at suitable intervals from the cultures growing at 37 C and assayed for alkaline phosphatase spectrophotometrically. The arrow indicates the time at which the cultures left log phase. (C and D). The cells were treated as in A and B with the exception that DL-α-glycerophosphate was the organic phosphate source and glycerol was added as the carbon source when P_i was supplied. WT medium containing the following additions was employed: 0.03 M glycerophosphate (○), 0.6% glycerol and 10^{-2} M P_i (□), 0.6% glycerol and 10^{-4} M P_i (△).*

whose products are involved in alkaline phosphatase regulation. Mutations at key steps in the pathway might lead to constitutive alkaline phosphatase synthesis. It is possible that the intracellular concentration of P_i directly affects alkaline phosphatase regulation; however, the recent experiments reported by Wilkins (24) do not support this idea. Wilkins observed that alkaline phosphatase synthesis could be obtained in the presence of excess medium P_i by starving cells for pyrimidines or guanine. Under these starvation conditions, no decrease in the internal P_i levels could be detected. These results, as well as the data presented in this paper, would suggest the involvement of an unknown product(s) derived from P_i in alkaline phosphatase regulation.

We have shown that the wild-type *phoT* gene is necessary if the *pst* system is to function in phosphate transport. This dependence has been demonstrated by P_i transport studies which reveal alterations in kinetic parameters of the *pst* system in *phoT* mutants, and by genetic studies which show the complete loss of the *pst* system and all P_i transport with the introduction of the *phoT* gene into a suitable genetic background (GR5154). Although these similarities suggest that *pst* and *phoT* might be the same gene, differences in the levels of constitutive alkaline phosphatase synthesis in P_i^- derivatives carrying *pst* or *phoT* mutations do not support this idea. When grown in media with 0.6% glycerol and 5×10^{-4} M DL-α-glycerophosphate, the *phoT*$^-$ derivative of GR5154 synthesizes 10 times the amount of alkaline phosphatase produced by the *pst*$^-$ derivatives of GR5154 used in this study.

In our experiments, the production of alkaline phosphatase by *phoT* mutants is to some extent a function of the external P_i concentration. The *phoT* mutants produce only one-third of the fully derepressed level of alkaline phosphatase when grown in media with 10^{-2} M P_i. This level is still at least 150 times the basal level found in the wild-type strain grown under these conditions. In these experiments, we have measured alkaline phosphatase production in media wherein the external P_i concentration was varied from 10^{-2} to 10^{-5} M. The maximal changes in alkaline phosphatase reduction occur in the range from 10^{-3} to 10^{-4} M P_i (Fig. 1).

Previous work in this field by Echols et al. (5) used 6×10^{-4} M as the high P_i concentration and 6×10^{-5} M as the low or limiting P_i concentration. In measuring alkaline phosphatase production in constitutive mutants, Echols et al. (5) observed a slight dependence on medium P_i concentration. However, only one strain, C14, exhibited as much as a twofold difference in alkaline phosphatase production in the high and low P_i media employed by these authors. In a later paper, Garen and Otsuji (9) observed a twofold change in alkaline phosphatase synthesis with the *phoS* mutant C86 and a 1.5-fold change in alkaline phosphatase synthesis with *phoS* mutants C31 and C105, using 10^{-3} M P_i as the high P_i concentration and P_i-starved cells for limiting P_i conditions.

The failure to observe similar changes in alkaline phosphatase levels with the other *phoS* and *phoT* mutants examined by Echols et al. (5) might simply result from the fact that 6×10^{-4} M P_i is not sufficiently high to ensure maximal repression. In addition, we have shown that mutant C112 only shows a threefold change in alkaline phosphatase production at different P_i concentrations when grown with glycerol and not with glucose as the carbon source. All of the experiments reported by Echols et al. were done in glucose-grown cells.

The involvement of the *phoS* gene product in P_i transport and alkaline phosphatase regulation remains obscure. We have shown that the *phoS*$^-$ derivative of GR5154 which is constitutive for alkaline phosphatase contains a functional *pst* P_i transport system. However, the *phoS*$^-$ cells are now Asi^s in contrast to the Asi^r parental strain.

Our genetic experiments on the effects of *phoS* and *phoT* mutants on P_i transport have been done with strains dependent on one P_i transport system, the *pst* system. However, there are at least four different phosphate transport systems in *E. coli* (2, 16; R. L. Bennett, Ph.D. thesis, Tufts Univ., 1972). A single strain may not have all of the P_i transport systems functioning at any given time. Jones (11) reported that the basal level of alkaline phosphatase in various *E. coli* strains shows the influence of uncharacterized genes. It is possible that some of his results stem from differences in P_i transport in the strains examined.

The overall scheme for alkaline phosphatase regulation in *E. coli* seems to combine elements of both positive and negative control. The positive requirement for the R1 protein for alkaline phosphatase induction is similar to the requirement for the C protein, product of the *araC* gene, for transcription of the arabinose operon of *E. coli* (6). In the case of the histidine operon of *Salmonella typhimurium*, which is regulated by negative control, constitutive synthesis of the enzymes of the histidine biosynthetic pathway can result from mutations at

several loci. Some of these constitutive mutations show altered synthesis or activation of histransfer ribonucleic acid, which plays a role in the repression of the histidine operon (15). Thus, mutations which only indirectly affect the synthesis of co-repressors can result in constitutive enzyme synthesis.

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