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Control of the Synthesis of Alkaline Phosphatase and the Phosphate-Binding Protein in *Escherichia coli*

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Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunological techniques, we have compared the synthesis of the *phoA* protein (alkaline phosphatase) and the *phoS* protein (phosphate-binding protein) in response to the level of phosphate in the medium in different genetic backgrounds containing the known alkaline phosphatase control mutations. Both proteins are produced in excess phosphate media in a *phoR1a*$^-$ strain, whereas neither protein is produced in a *phoB*$^-$ strain even under derepression conditions. In four different *phoR1c*$^-$ strains, however, the *phoA* product cannot be detected in extracts of cells obtained from any growth condition, whereas the *phoS* product is produced in both excess and limiting phosphate media. It is not yet known if *phoR1c*$^-$ mutants are a special class of mutations within the *phoB* gene or whether they occur in a separate cistron involved in alkaline phosphatase regulation. From these results we conclude that the expression of the *phoA* gene is not always co-regulated with expression of the *phoS* gene product. We have determined that the *phoS* protein is a component of periplasmic protein band P4 described by Morris et al. (1974). The *phoS* product lacks sulfur-containing amino acids and is extractable by treatment with polymyxin sulfate. The other component of band P4 contains methionine and/or cysteine and is not extracted by polymyxin sulfate treatment. Like the *phoS* and *phoA* proteins, its synthesis is sensitive to the concentration of phosphate in the growth medium. In addition, the existence of a new class of periplasmic proteins synthesized at maximum rate in high phosphate media is demonstrated.

The alkaline phosphatase (EC 3.1.3.1) of *Escherichia coli* was the first enzyme protein shown to be localized in the space between the cytoplasmic membrane and the cell wall (19). This space was later to be designated the periplasmic space (12). In this location alkaline phosphatase serves to scavenge inorganic phosphate (P_i) from phosphomonoesters external to the cytoplasmic membrane during periods of P_i growth limitation. The participation of P_i in the regulation of alkaline phosphatase synthesis has been recognized since the first descriptions of this enzyme more than 15 years ago (13, 25). In the wild-type strain, alkaline phosphatase synthesis is "repressed" in media containing excess P_i and "derepressed" in media limiting in P_i. The specific role of P_i in alkaline phosphatase regulation is still not known. Although internal P_i levels have been suggested to be of prime importance in regulation, recent studies have indicated that lowered internal P_i concentrations may not be directly related to alkaline phosphatase derepression. Wilkins (27) has shown that pyrimidine starvation in high P_i media also causes derepression. We have found that internal P_i levels are maintained at a constant high level at least 2 h after the cells enter the P_i-limited growth phase while alkaline phosphatase synthesis is proceeding at its maximal rate (unpublished data).

Several classes of constitutive mutations that synthesize alkaline phosphatase even during growth in excess P_i media have been described (8) and mapped at three distinct loci on the *E. coli* chromosome: *phoS* (1), *phoT* (8, 28), and *phoR* (3, 8). As a result of our studies on the mechanisms of P_i transport in *E. coli*, we have found that two of the loci leading to constitutive alkaline phosphatase production, *phoS* and *phoT*, seem to have a primary role in P_i transport and only an indirect role in alkaline phosphatase regulation (28). *phoS* is the structural gene for the phosphate-binding protein (11) originally designated the R2a protein (10). We have shown that there are two major P_i transport systems in *E. coli*, which are synthesized constitutively, PST and PIT (28). The PST system (coded for by a gene[s] mapping near min

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74 on the *E. coli* chromosome) has a K_m of 0.4 μM and a V_{max} of 5.3 nm P_i (optical density at 600 nm)$^{-1}$ (min)$^{-1}$; its activity is inhibited by NaCN but resistant to arsenate. The PIT system (coded for by gene[s] mapping in the *xyl-malA* region of the chromosome) has a K_m of 33 μM and a V_{max} of 15 nm P_i (optical density at 600 nm)$^{-1}$(min)$^{-1}$; its activity is resistant to NaCN but sensitive to arsenate (G. R. Willsky and M. H. Malamy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P192, p. 173). Loss of the phosphate-binding protein leads to changes in the substrate specificity of the P_i transport system designated PST (29). Mutations at *phoT* lead to the loss of P_i transport activity by the PST system (28) and are distinguishable from *pst* mutations by P1 transduction.

The *phoR* gene appears to be directly involved in the regulation of alkaline phosphatase synthesis. *phoR1b*$^-$ mutants are constitutive and fully derepressible (8), whereas *phoR1a*$^-$ mutants are partially constitutive but cannot synthesize fully derepressed alkaline phosphatase levels in limiting P_i media (8). This property of *phoR1a*$^-$ mutants first led to the suggestion that the *phoR* product is an inducer or plays a positive role in the control of alkaline phosphatase synthesis (9).

Further support for a positive control model of alkaline phosphatase regulation came from the properties of *phoR1c*$^-$ mutants mapping in the *phoR* region (8, 9); these mutants are unable to synthesize alkaline phosphatase under any growth conditions. *phoR1c*$^-$ mutants were shown by complementation and recombination studies to possess an intact transactivatable alkaline phosphatase structural gene (*phoA*$^+$). Recently, Kreuzer et al. (16) demonstrated that a *phoR1c*$^-$ mutant can complement with a *phoR1a*$^-$ mutant to restore the repressible phenotype. This result suggests that *phoR1a* and *phoR1c* are separate cistrons that may be adjacent.

The properties of *phoB*$^-$ mutants, a new class of alkaline phosphatase-negative mutations mapping apart from the *phoA* structural gene (4), also suggest a positive control model for alkaline phosphatase regulation. Recombination data suggest that the *phoB*$^-$ mutations are located in the same genetic region as *phoR* mutations, whereas complementation tests reveal that *phoB*$^-$ and *phoR1a*$^-$ mutations complement to restore the wild-type repressible phenotype (4). Using a series of specialized transducing phages carrying the *phoA* and/or *phoR-phoB* regions of the chromosome, Brickman and Beckwith (5) have recently demonstrated that a product(s) of the *phoR-phoB* region is required for alkaline phosphatase synthesis. The loss by deletion of the genetic region presumed to contain the *phoR* and *phoB* sites leads to the loss of alkaline phosphatase synthesis under all growth conditions.

While characterizing the *phoB*$^-$ mutants, Morris et al. (20) discovered several new periplasmic proteins whose synthesis, like that of alkaline phosphatase, is regulated by the level of P_i in the growth medium. *phoB*$^-$ mutants lack the *phoA* product as well as these other periplasmic proteins. The question of whether the *phoB*$^-$ and *phoR1c*$^-$ mutations are the same arises. Kreuzer et al. (16) assumed that they were in the same cistron and suggested renaming all *phoR1c*$^-$ mutations with a *phoB*$^-$ designation. However, in this communication, we show that *phoR1c*$^-$ and *phoB*$^-$ mutants, although failing to synthesize the *phoA* product, can be distinguished from each other by their effect on *phoS* synthesis. Thus, we will retain the original designations *phoR1c* and *phoB* until complementation studies are complete and the issue of whether *phoB* and *phoR1c* are in the same cistron is resolved.

In this paper we identify the product of the *phoS* gene, the phosphate-binding protein, as a component of periplasmic protein band P4 as defined by Morris et al. (20). We compare the synthesis of alkaline phosphatase and the phosphate-binding protein in response to the level of P_i in the growth medium and in genetic backgrounds containing the known alkaline phosphatase control mutations. This work will describe a newly recognized class of periplasmic proteins whose maximum synthesis occurs during growth in excess P_i medium.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study, with pertinent genotypes, are given in Tables 1 and 2.

Markers involved in aspects of P_i transport are as described in reference 28. PST and PIT are the two major constitutive P_i transport systems. All derivatives of Hfr Cavalli K10 are dependent on the PST P_i transport system since they contain a *pit*$^-$ mutation and lack the PIT P_i transport system. In several cases this *pit*$^-$ mutation has been transferred by conjugation into strains of the GR series. Mutations at *phoT* (isolated as alkaline phosphatase constitutive mutations [8]) or at *pst* (isolated as arsenate resistant and unable to grow with P_i as phosphate source [2]) lead to inactivation of the PST P_i transport system. Although *phoT*$^-$ and *pst*$^-$ mutations map in the vicinity of min 74 on the *E. coli* chromosome, these mutations are distinguishable by several criteria, including differences in their levels of alkaline phosphatase synthesis in high-phosphate medium (28). To use these strains for studies on constitutive P_i transport, we eliminated P_i transport by the inducible *glpT* permease system by introduc-

TABLE 1. *Bacterial strains from this laboratory*

Strain	Relevant genotype[a]				Source
	pst	*pit*	Known alkaline phosphatase control mutations	Other	
GR5154	+	−K10	+	*ilv*⁻	—[b]
GR5156	+	−K10	*phoS*⁻ (C86)	*ilv*⁺	—[c]
GR5167	[+]	−K10	*phoT*⁻ (C90)	*ilv*⁺	—[c]
GR5158	+	−K10	*phoS*⁻ (C72)	*ilv*⁺	—[c]
GR5159	−UR3	−K10	+	*ilv*⁺	—[c]
GR5160	+	−K10	*phoS*⁻ (C78)	*ilv*⁺	—[c]
GR5134	+	+	+		—[d]
GR5205	+	−K10	+	*strA*ʳ *mtl*⁻	—[e]
GR5211	+	+	+	*lacZ*⁻ *proC*⁺	—[e]
GR5198	+	+	*phoS*⁻ (C86)		—[f]
GR5226	+	+	+	*lac*⁺ *proC*⁻	—[g]
GR5230	+	+	*phoR1a*⁻ (C2)	*lac*⁺ *proC*⁺	—[h]
GR5232	+	+	*phoR1c*⁻ (S3)	*lac*⁺ *proC*⁺	—[i]
GR5238	+	+	*phoB*⁻ (LEP1)	*lac*⁺ *proC*⁺	—[j]
LG5	+	+	*phoB*⁻ (LEP1)	*lac*⁺ *proC*⁺	—[j]

[a] The conventions of Demerec et al. (7) have been used to describe cell genotypes, except we designate mutant alleles as minus (*phoS*⁺ is the wild-type strain and *phoS*⁻ is a mutant). The nomenclature incorporated into the *E. coli* map (23) has also been followed. Only the portions of the genotype relevant to this study are listed for each strain. All strains constructed for this study have the F⁻ mating type and contain a *glpT*⁻ mutation.

[b] GR5154 is used as the strain dependent upon the PST constitutive P_i transport system. The construction of this strain has been previously described (28).

[c] The construction of these strains by P1 transduction, using GR5154 as the recipient and selecting for *ilv*⁺ transductants, has been previously described (28, Table 3).

[d] Strain GR2131 was constructed from strain 13.6, as described in reference 28, by a series of manipulations that included: (i) introduction of *phoA*⁻ from U7; (ii) introduction of *glpT*⁻ from UR13; and (iii) introduction of *phoA*⁺ from XS1. Strain GR5134 was selected as a spontaneous *bgl*⁺ (β-glucoside utilization [28]) colony on MacConkey salicin plates from strain GR2131.

[e] These strains were constructed from a conjugation between UR1 (*pst*⁻ *pit*⁻ *bgl*⁻ *strA*ˢ *mtl*⁺) and GR5134 using a *str*ᴿ *mtl*⁺ selection. GR5211 was verified to be an exconjugant similar to the parental GR5134 (*pst*⁺ *pit*⁺), and GR5205 was verified to be an exconjugant containing only the PST P_i transport system (*pst*⁺ *pit*⁻).

[f] Strain GR5198 was constructed by P1 transduction from strain GR2131 (see footnote *d* and reference 28). A spontaneous *bgl*⁺ colony of *phoS*⁻ strain C86 was the donor strain. The selection was for *bgl*⁺ on minimal medium plates containing salicin as the carbon source and 1 mM L-α-glycerophosphate as the phosphate source. The presence of the *phoS*⁻ gene was scored using the plate assay for constitutive alkaline phosphatase synthesis (28).

[g] GR5226 was constructed by P1 transduction with GR5211 as the recipient and CA7087 as the donor. The selection was for *lac*⁺, and a *proC*⁻ strain was detected by replica plating.

[h] GR5230 was constructed by P1 transduction using GR5226 as the recipient and C2 as the donor strain. The selection was for *pro*⁺, and an alkaline phosphatase constitutive transductant was detected as described (28).

[i] GR5232 was constructed as described in footnote *h* except that S3 was the donor strain, and an alkaline phosphatase-negative strain was detected as described (28).

[j] GR5238 was constructed by transducing strain GR5226 to *pro*⁺ using P1 grown on a donor strain containing the *proC phoB lac* chromosomal region from strain LG5. LG5 was selected as a *pro*⁺ transductant using AB3311 as the donor strain and LEP1 as the recipient. A *pro*⁺ *phoB*⁻ *lac*⁺ transductant was detected by replica plating.

ing a *glpT*⁻ mutation. In spite of the absence of the *glpT* transport system, strain GR5159 and other derivatives lacking both the PST and PIT transport systems can grow on L-α-glycerophosphate as a phosphate source but not as a carbon and phosphate source (28).

Chemicals. Polymyxin B sulfate, egg white lysozyme, and DL-α-glycerophosphate were obtained from Sigma Chemical Corp. Acrylamide was from Eastman Organic Co. Tris(hydroxymethyl)aminomethane (Tris) was from Research plus Labs. Ion exchange resin AG501-X8 (20 to 50 mesh), bisacrylamide, and TEMED (*N,N,N',N'*-tetramethylethylenediamine) were from Bio-Rad Laboratories. [^{35}S]methionine (250 Ci/mmol) and uniformly ^{14}C-labeled amino acids (120 to 400 mCi/mmol) were obtained from New England Nuclear Corp.

Media. The minimal medium used was WT (2) supplemented with 2 mg of thiamine per liter. After autoclaving, sterile glycerol was added as the car-

TABLE 2. *Bacterial strains from other laboratories*[a]

Strain	Mating type	Alkaline phosphatase control mutations	Other	Source
LEP1	F⁻	*phoB*⁻	*lac*⁻ *proC*⁻	E. Yagil (4)
G206	HfrC	*phoR1c*⁻		A. M. Torriani (16)
H2	HfrC	*phoR1c*⁻		A. M. Torriani (16)
H9	HfrC	*phoR1c*⁻		A. M. Torriani (16)
S3	HfrC	*phoR1c*⁻		A. M. Torriani (16)
C2	HfrC	*phoR1a*⁻		A. M. Torriani (16)
CA7087	HfrH	+	*proC*⁻	J. Beckwith
AB3311	Hfr Rl	+	*proC*⁺	H. Rosenberg
XPh1a	F⁻	*phoR1a*⁻ (C2)		J. Beckwith (5)
XPh24	F⁻	Δ(*brnQ phoA proC phoB*)		J. Beckwith (5) (see text)

[a] See footnote *a*, Table 1.

bon source to a final concentration of 0.6%. Amino acids (proline, leucine, and tryptophan) and adenine were added to a final concentration of 5 mg/liter. Isoleucine and valine were added to a final concentration of 5 mg/liter when required. A stock P_i solution was prepared from anhydrous sodium phosphate, adjusted to pH 7.5 with sodium hydroxide and sterilized by autoclaving. DL-α-Glycerophosphate solutions were sterilized by filtration through 0.45-μm membrane filters (Millipore Corp.). The following phosphate sources were used: GP medium contained 1.0 mM DL-α-glycerophosphate; high P_i medium contained 1 mM inorganic phosphate; and low P_i medium contained 0.1 mM inorganic phosphate. Alkaline phosphatase production was repressed in GP and high P_i media and derepressed after growth in low P_i medium or after starvation. P_i-starved cells were prepared by harvesting cells during exponential growth from GP medium by centrifugation at 4 C, washing once with unsupplemented WT medium, and resuspending in supplemented WT medium without a phosphate source. Incubation was continued at 37 C for 2 h.

P1 transduction. The procedure for P1 transduction and the media used for these experiments have been previously described (28).

Isolation of periplasmic proteins. Periplasmic proteins were isolated from cells growing at 37 C under conditions of alkaline phosphatase repression or derepression using the following procedures. All centrifugations were performed at 4 C.

(i) Osmotic shock. The osmotic shock procedure of Nossal and Heppel (21) was used. A 500-ml portion of cells was harvested by centrifugation at 13,000 × *g*, washed twice in 0.01 M Tris-hydrochloride (pH 7.5), 0.03 M NaCl, and resuspended in 40 ml of 0.033 M Tris-hydrochloride (pH 7.5). A 40-ml amount of stage I buffer (40% sucrose, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 0.033 M Tris-hydrochloride [pH 7.5]) was added with rapid mixing, and the suspension was left at room temperature for 10 min. The cells were then collected by centrifugation at 13,000 × *g* for 10 min, rapidly resuspended in 80 ml of stage II solution (0.5 mM $MgCl_2$), and stirred rapidly while kept in an ice bath at 0 C for 10 min. The shocked cells were then removed by centrifugation at 13,000 × *g* for 10 min, and the supernatant containing the periplasmic proteins was decanted and saved. This shockate was lyophilized and resuspended in 1 ml of 0.5 M Tris-hydrochloride (pH 7.5). The final shockate also contained 40 mM $MgCl_2$ from the stage II buffer.

(ii) EDTA-lysozyme treatment. The EDTA-lysozyme procedure of Morris et al. (20) was followed. Cells were harvested by centrifugation at 12,000 × *g* and washed in cold buffer (10 mM Tris-hydrochloride [pH 7.5], 1 mM $MgCl_2$). The cell pellet was then resuspended in 1/60 of the original volume of a solution containing 25% sucrose, 10 mM Tris-hydrochloride (pH 7.5), 0.5 mg of lysozyme per ml, and 2 mM EDTA (pH 8.0). After 20 min at room temperature, the cell suspension was centrifuged at 13,000 × *g* for 10 min and the supernatant was saved. This periplasmic protein fraction was lyophilized and resuspended in distilled water to one-half of the original starting volume (and also contained 50% sucrose, 20 mM Tris-hydrochloride [pH 7.5], 2 mM EDTA [pH 8.0], and 1 mg of lysozyme).

(iii) Polymyxin sulfate treatment. The polymyxin sulfate procedure of Teuber and Cerny (24) was followed with some modifications. A total of 50 ml of cells was harvested by centrifugation at 12,000 × *g* for 10 min, washed once in 10 mM Tris-hydrochloride (pH 7.5), 1 mM $MgCl_2$, and 10 μM $ZnCl_2$, and resuspended in 1 ml of the same buffer. This cell suspension was incubated with 1 mg of polymyxin sulfate at 37 C for 30 min and then centrifuged at 12,000 × *g* for 10 min. The supernatant was lyophilized and resuspended in 0.3 ml of water (and also contained 33 mM Tris-hydrochloride [pH 7.5], 3.3 mM $MgCl_2$, 33 μM $ZnCl_2$, and 1 mg of polymyxin).

Radioactive labeling of periplasmic proteins. In certain cases, the cell suspensions were labeled with uniformly ^{14}C-labeled amino acids (15 μCi/ml; without added carrier) or [^{35}S]methionine (20 μCi/ml; specific activity, adjusted to 2.7 μCi/μg with cold methionine) for 20 min just prior to harvesting. Incorporation of radioactivity, as measured by trichloroacetic acid-precipitable counts, was linear over the 20-min period.

Immunodiffusion. Immunodiffusion studies were performed with Ouchterlony slides (14) containing 4 ml of 0.8% agarose in water with trace amounts of merthiolate. The pattern used 10 μl of antibody in

the central well and 5 μl of antigen in the outer wells. Precipitin lines were allowed to develop overnight in a humid chamber at room temperature. Antibody to the phosphate-binding protein was kindly provided by R. G. Gerdes and H. Rosenberg (11). The preparation of antibody to crystalline alkaline phosphatase has been previously described (M. H. Malamy, Ph.D. thesis, New York Univ., New York, 1963). Varying amounts of the periplasmic protein samples were used as antigens as indicated in the figure legends.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis used the discontinuous buffer system and apparatus described by Studier (22). Spacer gels contained 0.063 M Tris-hydrochloride (pH 6.8), 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, and 5% acrylamide. Separating gels contained 0.19 M Tris-hydrochloride (pH 8.8), 0.1% SDS, 2 mM EDTA, and 10% acrylamide. Polymerization was initiated with TEMED and ammonium persulfate. The acrylamide-bisacrylamide ratio was 30:0.8. The electrode buffer contained 0.05 M Tris, 0.38 M glycine, 0.1% SDS, and 2 mM EDTA and was adjusted to pH 8.3 if necessary with NaOH. The periplasmic protein sample (10 to 25 μg) was added to the appropriate buffer concentrate to give a final solution containing the sample in 0.6 M Tris-hydrochloride (pH 8.0), 1% SDS, 10% glycerol, 1% mercaptoethanol, and 2 mM EDTA. The sample was then placed in a boiling-water bath for 2 min. From 10 to 25 μl of sample was added to each gel slot in the 1.5-mm slab gel so that the protein content of each slot would be ~25 μg. For samples of periplasmic proteins obtained by the EDTA-lysozyme treatment, glycerol was omitted from the boiling buffer, since sucrose was already present in these samples. The best results in this procedure were obtained with glycine and SDS from Sigma Chemical Co. Acrylamide was purified by passage through AG501-X8 resin. Electrophoresis was performed either at 25 V (constant) for 15 h or at 125 V (constant) for 2 h at room temperature. Pyronin Y was used as the tracking dye. At the end of the run the protein bands were visualized by either of the following procedures. When radioactive samples were used, the slab was transferred to Whatman no. 1 filter paper and dried under vacuum while placed over a steaming water bath. The dried gel was then exposed to Kodak No-Screen X-ray film for 3 to 20 days. For non-radioactive samples the gel slab was first placed in Coomassie blue stain (2.5 g of Coomassie blue per liter, 45% methanol, 9% acetic acid) for 1 h (26) and then in Coomassie copper stain (0.5 g of Coomassie blue per liter, 0.5 g of $CuSO_4 \cdot 5H_2O$ per liter, 9% acetic acid, 45% methanol) for 1 h. The gel was destained by frequent changes of 7.5% acetic acid, 5% methanol, using slight agitation.

Assays. The quantitative assay for alkaline phosphatase using *p*-nitrophenylphosphate as the substrate has been previously described (28). A unit of alkaline phosphatase specific activity is 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. Protein concentrations were determined by the method of Lowry et al. (18). Bovine serum albumin was used as the protein standard.

RESULTS

Repression of alkaline phosphatase by "excess" L-α-glycerophosphate. We first examined the repression and derepression of alkaline phosphatase and other periplasmic proteins in a series of strains constructed for studies on P_i transport (28). Some of these strains were P_i transport deficient and unable to utilize P_i for growth. Therefore, the usual method of repression by high levels of P_i in the media was not applicable to these strains. We had previously observed that the phosphate requirement of these P_i transport-defective mutants could be satisfied by L-α-glycerophosphate. In addition, we had shown that repression conditions could be obtained by growing wild-type cells containing the L-α-glycerophosphate permease system (*glpT*$^+$) (17) on L-α-glycerophosphate as sole carbon and phosphate source (28). However, all the strains in this present study also lacked the *glpT* permease system but could still use L-α-glycerophosphate as a phosphate source but not as a carbon source. The mechanism of L-α-glycerophosphate transport in these *glpT*$^-$ strains is not clear; there may be sufficient *glpT* activity remaining to provide the small quantities of L-α-glycerophosphate required as a phosphate source, or there may be another transport system capable of transporting small amounts of L-α-glycerophosphate into the cell.

Table 3 compares the levels of alkaline phosphatase synthesis in three parental strains, GR5154 and GR5205 (both *pst*$^+$, *pit*$^-$), GR5211 (*pst*$^+$,*pit*$^+$), and representatives of some classes of alkaline phosphatase control mutations after growth in limiting P_i medium (0.1 mM P_i) and excess phosphate medium (either 1 mM P_i or 1 mM L-α-glycerophosphate). Although there was a great deal of variability from experiment to experiment in the amount of alkaline phosphatase synthesized by wild-type strains in 1 mM L-α-glycerophosphate medium, there was at least a 20- to 30-fold repression of alkaline phosphatase synthesis when compared with the fully derepressed values (0.1 mM P_i) obtained in the same experiment. In spite of the variability, to examine all strains in the same excess phosphate conditions we routinely used 1 mM L-α-glycerophosphate-containing medium as repression medium.

Strains containing the constitutive mutations *phoS*$^-$ and *phoR1a*$^-$ produced high levels of alkaline phosphatase in both excess phosphate media. The *pst*$^-$, *pit*$^-$ mutant GR5159

TABLE 3. *Alkaline phosphatase synthesis in limiting phosphate and excess organic and inorganic phosphate media*

Strain	Genotype	Alkaline phosphatase sp act[a]		
		Glycerol + L-α-glycerophosphate (1 mM)	Glycerol + 1 mM P_i	Glycerol + 0.1 mM P_i
GR5154	*pst*$^+$ *pit*$^-$	0.05[b] (0.05-0.3)[c]	<0.01[b]	2.2[b] (2.2-12.3)
GR5156	*pst*$^+$ *pit*$^-$ *phoS*$^-$	1.79 (1.8-4.0)	0.87 (0.99)	2.2 (0.69)
GR5159	*pst*$^-$ *pit*$^-$	0.57 (0.35-0.7)		
GR5205	*pst*$^+$ *pit*$^-$	0.02 (0.02-0.14)	<0.01	2.3 (2.3-4.0)
GR5211	*pst*$^+$ *pit*$^+$	0.02 (0.02-0.9)	<0.01	2.2 (2.2-9.4)
GR5230	*pst*$^+$ *pit*$^+$ *phoR1a*$^-$	0.78 (0.22)	0.55 (0.28)	0.88 (0.34)
GR5232	*pst*$^+$ *pit*$^+$ *phoR1c*$^-$	<0.01 (0.02)	<0.01 (<0.02)	0.03 (0.02)
GR5238	*pst*$^+$ *pit*$^+$ *phoB*$^-$	<0.01 (<0.02)	<0.01 (<0.02)	<0.02 (<0.02)

[a] Cells were grown to stationary phase in the indicated medium, washed once with 0.5 M Tris-hydrochloride (pH 8.0), and treated for 30 min at 37 C with 0.1 ml of toluene per ml of cell culture. Alkaline phosphatase was assayed quantitatively as described in Materials and Methods.

[b] All reported values in these columns are from cultures grown on the same day, and each number represents the average of two independent enzyme assays.

[c] The numbers in parentheses are the range of values observed in at least four experiments except for strains GR5156, GR5230, GR5232, and GR5238, which were only repeated once.

synthesized about 25% of the fully derepressed wild-type level of alkaline phosphatase in excess L-α-glycerophosphate medium. It is not clear whether this is true constitutive synthesis or whether this strain is experiencing P_i limitation as a result of diminished P_i transport ability. The *phoB*$^-$ and *phoR1c*$^-$ strains did not produce significant amounts of alkaline phosphatase in either excess or limiting phosphate media.

Identification of the phosphate-binding protein in osmotic shockates. Periplasmic proteins were prepared by the osmotic shock method of Nossal and Heppel (21) from cultures growing in excess L-α-glycerophosphate medium (repression conditions) and from cultures starved of all phosphate for 2 h (derepression conditions). This is the derepression protocol used by Garen and Otsuji (10) and by Morris et al. (20). The shockates were lyophilized, resuspended in 0.5 M Tris-hydrochloride (pH 7.5), analyzed on SDS-polyacrylamide gels, and stained with Coomassie blue. In Fig. 1 and in all other gels of periplasmic proteins in this paper, we have adopted the nomenclature of Morris et al. (20) for periplasmic proteins synthesized in limiting phosphate medium. Band P1, corresponding to alkaline phosphatase, was produced only under conditions of phosphate limitation in the parental strain GR5154 (Fig. 1). In the *phoT*$^-$ and *phoS*$^-$ derivatives, however, band P1 was produced in excess and limiting phosphate media. In the *pst*$^-$ derivative GR5159, band P1 is poorly visible in this gel. Table 3 indicates, however, that this strain synthesizes about 25% of the fully derepressed level of alkaline phosphatase in excess L-α-glycerophosphate medium. In other gels, or with higher protein concentrations, band P1 is clearly visible in extracts of *pit*$^-$, *pst*$^-$ strains grown in excess L-α-glycerophosphate or after phosphate starvation.

Band P2 is found in wild-type extracts only after phosphate limitation but is synthesized in excess and limiting phosphate conditions by the *phoS*$^-$,*phoT*$^-$ and *pst*$^-$ derivatives.

Band P3, as identified by Morris et al. (20), is not visible in the samples used here. The appearance of this band is reported to be highly variable (20), and factors influencing its presence are not known.

Protein at the band P4 position was produced by the wild-type strain only after phosphate starvation. Band P4 is present in extracts of *phoT*$^-$ and *pst*$^-$ cells obtained from excess and limiting phosphate conditions. Protein with the mobility of band P4 is greatly diminished or missing in the *phoS*$^-$ extracts. Morris et al. (20) have reported that periplasmic protein bands P1 through P4 are present in EDTA-lysozyme extracts obtained from a *phoS*$^-$ mutant. Yet the ^{35}S-labeled periplasmic protein described by Morris et al. (20) at position P4 cannot be the *phoS* product, since the phosphate-binding protein lacks methionine and cysteine (10, 11). These results suggest that band P4 consists of at least two periplasmic proteins whose synthesis is regulated by the level of phosphate in the medium: the phosphate-binding protein and another unidentified sulfur-containing protein(s).

Periplasmic protein band P4 consists of the phosphate-binding protein and another protein whose synthesis is controlled by the level

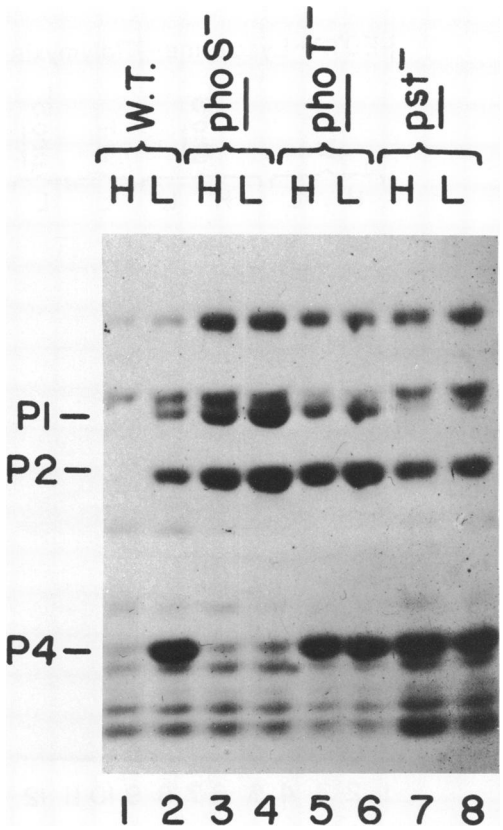

FIG. 1. *Identification of the phosphate-binding protein in osmotic shockates from E. coli. All gel samples contained 25 μg of periplasmic protein prepared by the osmotic shock procedure. The SDS-10% polyacrylamide gel was stained with Coomassie blue. Odd-numbered slots contain samples obtained from cells growing in excess L-α-glycerophosphate (GP) medium, and even-numbered slots contain samples from cells subjected to phosphate starvation conditions. GR5154 was the wild-type strain pst⁺ pit⁻ phoS⁺ phoT⁺ (slots 1 and 2); GR5156 was the phoS⁻ strain (slots 3 and 4). GR5157 was the phoT⁻ strain (slots 5 and 6), and GR5159 was the pst⁻ strain (slots 7 and 8).*

of phosphate in the medium. Different methods of preparing periplasmic protein samples often release different classes and varying amounts of these proteins (6, 12). Cerny and Teuber (6) have reported that polymyxin B sulfate treatment of *E. coli* cells releases less of the periplasmic proteins than other commonly used procedures. They mention, however, that the phosphate-binding protein is one of the proteins extracted by polymyxin sulfate treatment (H. Rosenberg as quoted in reference 6). We therefore used polymyxin sulfate treatment to further analyze the nature of the proteins in band P4. To eliminate any variations that may have arisen from our use of the osmotic shock procedure, we also repeated the extraction of periplasmic proteins using the EDTA-lysozyme procedure of Morris et al. (20).

Periplasmic proteins were labeled with [^{35}S]methionine or ^{14}C-labeled amino acids and extracted by both polymyxin sulfate treatment and the EDTA-lysozyme procedure. In Fig. 2a, Coomassie blue was used to stain an SDS-polyacrylamide gel of labeled periplasmic proteins obtained from the wild-type and a *phoS*⁻ strain. In the case of samples prepared by EDTA-lysozyme treatment, the following observations can be made. (i) The wild-type strain produced alkaline phosphatase (band P1) and band P2 only under conditions of phosphate starvation. (ii) the *phoS*⁻ mutant produced both band P1 and band P2 in excess and limiting phosphate conditions. (iii) Band P4 was synthesized in the wild-type strain only during phosphate limitation. (iv) Some band P4 is produced by the *phoS*⁻ mutant in excess phosphate growth conditions, but much larger amounts appear in extracts prepared from phosphate-limited cells.

In the case of extracts prepared by polymyxin sulfate treatment, although small amounts of bands P1 and P2 are released from the wild-type strain during phosphate limitation, large amounts of band P4 can be detected. By contrast, the *phoS*⁻ mutant extracts lack any significant material with the mobility of band P4, whereas they contain large amounts of bands P1 and P2. From these results we can conclude that band P4 consists of at least two proteins that are produced maximally during limited phosphate growth conditions. Only one of these proteins was extracted from the cell by polymyxin sulfate treatment. We will refer to the polymyxin-extractable protein as band P4a and the second protein of band P4 as P4b. The absence of band P4a in polymyxin sulfate extracts of the *phoS*⁻ mutant C86, known not to produce cross-reacting material (10), allows us to conclude that band P4a is the *phoS* product, the phosphate-binding protein.

Figure 2b shows an autoradiogram of a gel prepared in the same manner and with the same samples used for Fig. 2a. Each strain is presented twice with ^{14}C-labeled amino acid-labeled samples at the left and [^{35}S]methionine-labeled samples at the right. In the case of periplasmic proteins prepared by EDTA-lysozyme treatment, comparison of the stained gel and the autoradiogram reveals the same series of bands with the exception of bands Pα, Pβ, and Pγ, which were radioactively labeled only during growth in high-phosphate conditions

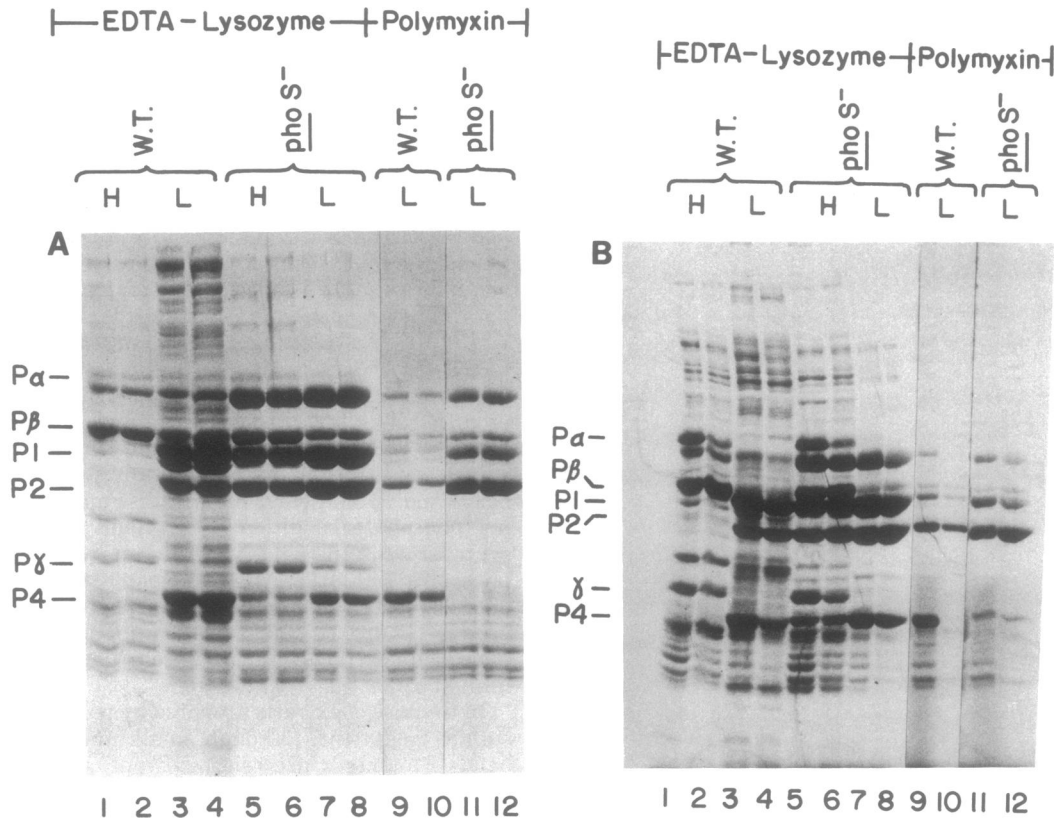

FIG. 2. *Periplasmic protein band P4 consists of the phosphate-binding protein and another protein whose synthesis is controlled by the amount of phosphate in the medium. All slots contain 10 to 25 μg of protein and 2 × 10³ to 10⁴ counts/min of ^{14}C or ^{35}S. Each sample is presented in duplicate; the odd numbers were labeled with ^{14}C-labeled amino acids and the even numbers are labeled with [^{35}S]methionine. SDS-10% polyacrylamide gels were used. Samples 1 through 8 were obtained by EDTA-lysozyme treatment (1, 2, 5, and 6 were obtained from high-phosphate-grown cells [GP medium]; 3, 4, 7, and 8 were obtained from phosphate-starved cells). Samples 9 through 12 were obtained by polymyxin sulfate treatment of phosphate-starved cells. In gel samples prepared from polymyxin sulfate extracts, a significant amount of protein remained with most of the polymyxin sulfate at the origin. GR5154 (1-4, 9, 10) was the wild type-strain and GR5156 (5-8, 11-12) was the phoS⁻ mutant. (a) This picture was made from a gel stained with Coomassie blue (see Materials and Methods). (b) This autoradiogram was made by exposing a dried gel to Kodak No-Screen film for 20 days.*

(see below). However, for the case of periplasmic proteins prepared by polymyxin sulfate treatment, a major difference was apparent when comparing the stained gel and the autoradiogram. Although protein with the mobility of band P4 was present in the stained gel in both samples obtained from phosphate-starved, wild-type cells, material at the band P4 position was only seen on the autoradiogram of the ^{14}C-labeled amino acid-labeled sample. Incubation of the phosphate-starved, wild-type cells with [^{35}S]methionine followed by treatment with polymyxin sulfate did not result in the release of a labeled protein with the properties of band P4. In addition, when phosphate-starved *phoS*⁻ cells were treated with polymyxin sulfate, there was a total absence of material at the band P4 position in both the stained gel and the autoradiogram.

These results show that there are at least two proteins in band P4 whose synthesis is regulated by the concentration of phosphate in the medium. One of these proteins (P4a) is the phosphate-binding protein, which can be released by both EDTA-lysozyme and polymyxin sulfate treatment but cannot be radioactively labeled with [^{35}S]methionine. The second protein (P4b), whose function is unknown, is only released by EDTA-lysozyme treatment and can be labeled with [^{35}S]methionine.

Immunological evidence for the absence of the phosphate-binding protein in a *phoS*⁻ mu-

tant. To demonstrate that the component of band P4 present in EDTA-lysozyme extracts of a *phoS*$^-$ mutant is not related to the phosphate-binding protein, immunological methods were used. Figure 3 shows an Ouchterlony double-diffusion slide of EDTA-lysozyme periplasmic protein extracts obtained from the wild-type strain and the *phoS*$^-$ mutants GR5156 and GR5158 grown with excess phosphate or after phosphate starvation. Using antibody to alkaline phosphatase, it can be seen in Fig. 3a that, for cells grown in excess phosphate, only the *phoS*$^-$ extracts contained alkaline phosphatase antigen, whereas under phosphate starvation conditions both the wild-type and the *phoS*$^-$ mutant extracts contained the antigen. Using antibody prepared against the phosphate-binding protein (Fig. 3b), it was apparent that the wild-type strain grown under phosphate starvation conditions produced large quantities of the phosphate-binding protein, whereas no detectable antigen was present in extracts prepared from phosphate-starved *phoS*$^-$ cells. (There was a minor contaminant [unrelated to the phosphate-binding protein] present in the original antigenic material used by Gerdes and Rosenberg [11], which is probably the cause of the spur seen in precipitin lines on double-diffusion plates containing this antiserum and highly concentrated extracts.) Even when grown with excess phosphate, the extract from the wild-type strain contained small amounts of the *phoS* antigen (very faint precipitin line), whereas the *phoS*$^-$ mutant extracts showed no precipitin line. Thus, the component of band P4 present in EDTA-lysozyme periplasmic ex-

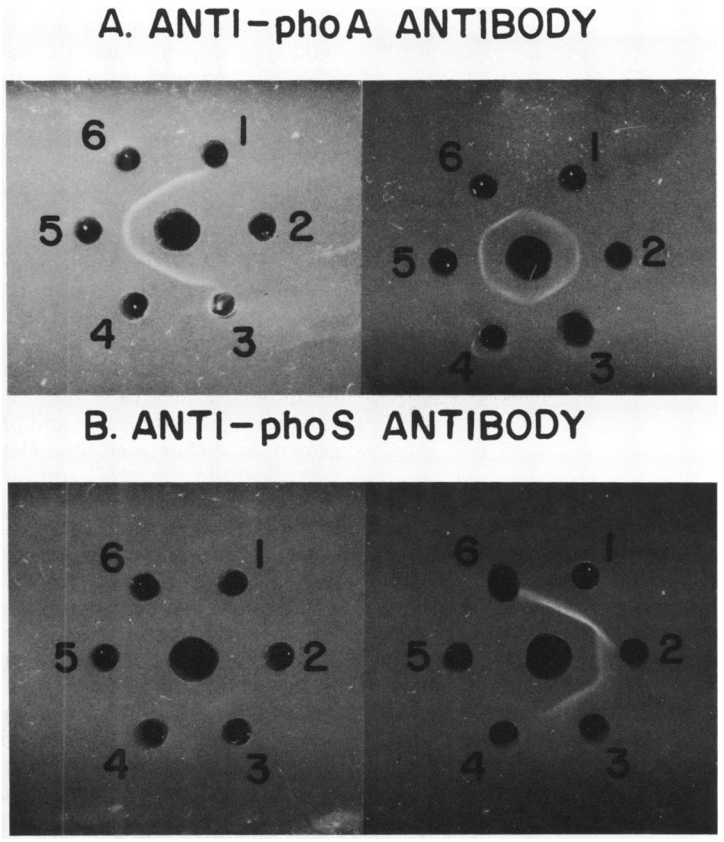

FIG. 3. *Immunological evidence for the absence of the phosphate-binding protein in a phoS$^-$ mutant. The antigen wells contained EDTA-lysozyme periplasmic protein samples: (1) GR5154, wild type (pst$^+$ pit$^-$), 30 μg; (2) GR5154, 5 μg; (3) GR5134, wild type (pst$^+$ pit$^+$), 25 μg; (4) GR5156 phoS$^-$ (pst$^+$ pit$^-$), 25 μg; (5) GR5156 phoS$^-$, 5 μg; (6) GR5158 phoS$^-$ (pst$^+$ pit$^-$), 28 μg. Samples on the left were prepared from high-phosphate-grown cells (GP medium), whereas the right-hand wells contained periplasmic protein isolated from phosphate-starved cells. In (a) the center well contained 10 μl of a 1:10 dilution of antiserum to alkaline phosphatase. In (b) the central well contained 10 μl of a 1:10 dilution of antiserum to the phoS phosphate-binding protein.*

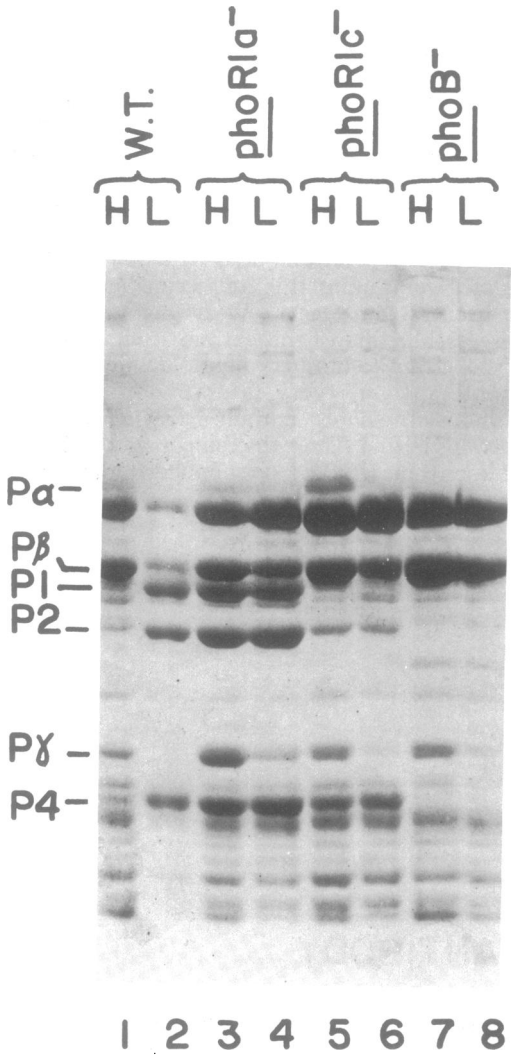

FIG. 4. *Analysis of periplasmic proteins obtained from cells with alkaline phosphatase control mutations. All samples on this SDS-10% polyacrylamide gel contained 10 to 20 μg of periplasmic protein prepared by EDTA-lysozyme treatment. Odd-numbered samples were from high-phosphate (GP) medium, whereas even-numbered samples were from phosphate starvation conditions. GR5134 was the wild-type strain, GR5230 was the phoR1a⁻ mutant, GR5232 was the phoR1c⁻ mutant, and GR5238 was the phoB⁻ mutant.*

tracts of a *phoS*⁻ strain is not capable of reacting with antiserum against the phosphate-binding protein.

Analysis of periplasmic proteins in cells containing alkaline phosphatase control mutations. Numerous control mutations affecting the synthesis of alkaline phosphatase have been described. Two classes of mutations, *phoR1a*⁻ and *phoR1b*⁻, result in constitutive synthesis of alkaline phosphatase (8), whereas two other classes of mutations, *phoB*⁻ and *phoR1c*⁻, result in greatly reduced amounts of alkaline phosphatase synthesis in any growth conditions (4, 8). We constructed an isogenic series of strains containing the various alkaline phosphatase control mutations starting with a wild-type strain, GR5211, containing both the PST and PIT phosphate transport systems (*pst*⁺,*pit*⁺). Periplasmic proteins were prepared by the EDTA-lysozyme procedure from cells after growth in L-α-glycerophosphate (GP) medium to achieve repression conditions and after 2 h of phosphate starvation to achieve derepression conditions. Figure 4 presents an SDS-polyacrylamide-stained gel of these samples. As has been noted in Fig. 1, band P1 (alkaline phosphatase) was produced only under conditions of phosphate starvation in the wild-type strain. Band P1 was present in the *phoR1a*⁻ extracts obtained after growth in excess or limiting phosphate, but it was missing in extracts of the *phoR1c*⁻ and *phoB*⁻ strains grown in either condition. Band P2 appears to follow the same pattern of regulation as band P1. Without additional evidence it is not possible to determine if the small amount of protein at the P2 position in the *phoR1c*⁻ extracts is the same P2 protein observed in the wild-type cells grown in limiting phosphate medium. As has been noted in Fig. 1, band P4 was produced only under conditions of phosphate limitation in the wild-type strain. Band P4 was present in extracts of the *phoR1a*⁻ and *phoR1c*⁻ mutants grown in either medium. However, band P4 was absent in the *phoB*⁻ mutant extract grown in either medium. In summary, a component(s) of band P4 that is normally controlled by the level of phosphate in the medium is synthesized in a cell containing a *phoR1c*⁻ mutation, whereas there is no evidence for the presence of the *phoA* protein in these cells.

Immunological evidence that a *phoR1c*⁻ strain that does not produce alkaline phosphatase produces the *phoS* protein. Figure 5 shows an Ouchterlony double-diffusion slide of the same periplasmic protein samples analyzed by SDS-polyacrylamide electrophoresis in Fig. 4 reacted with antisera to the phosphate-binding protein (Fig. 5a) and to alkaline phosphatase (Fig. 5b). In Fig. 5a it can be seen that extracts from either excess or limiting phosphate cultures of both the *phoR1a*⁻ and *phoR1c*⁻ strains contained the *phoS* protein. Figure 5b shows that alkaline phosphatase antigen was produced in the *phoR1a*⁻ strain in both excess or limiting phosphate conditions.

These immunological results confirm that at least the phosphate-binding protein component of band P4 seen on acrylamide gels is synthesized by a strain carrying the *phoR1c*$^-$ alkaline phosphatase control mutation. Using the present immunological assay, the same amount of *phoS* protein appears to be produced by the *phoR1c*$^-$ strain in excess and limiting phosphate media. However, the sensitivity of the assay is such that the amount of protein produced in the two media might vary by a factor of five.

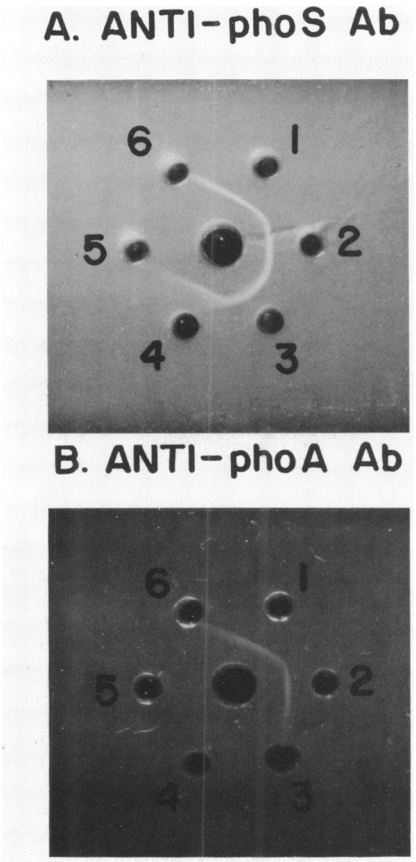

FIG. 5. *Immunological evidence that a phoR1c*$^-$ *strain that does not produce alkaline phosphatase does produce the phoS protein. The antigens present in the outer wells were the same periplasmic protein fractions used in Fig. 4. In (a) the center well contained a 1:10 dilution of antibody to the phosphate-binding protein, and in (b) the center well contained a 1:10 dilution of antibody to alkaline phosphatase. The numbers in parentheses represent the amount of protein in each antigen well: GR5230 (phoR1a*$^-$*)–1 (6 μg), 2 (8 μg); GR5232 (phoR1c*$^-$*)–3 (3.3 μg), 4 (3.5 μg); GR5238 (phoB*$^-$*)–5 (3.8 μg), 6 (4.0 μg). Odd-numbered wells contained protein fractions from high-phosphate cells (GP), whereas even-numbered wells contained protein fractions from phosphate-starved cells.*

Periplasmic proteins produced at maximum rate in high-phosphate medium. Comparison of autoradiograms of periplasmic proteins labeled with ^{14}C-labeled amino acids in excess phosphate growth medium and after phosphate starvation has clearly revealed the presence of three protein bands, which appear to be synthesized at maximum rate in excess phosphate medium (Fig. 2b). This distinction is difficult to make on the basis of the stained gel (Fig. 2a) but is clear in the autoradiogram presented in Fig. 2b. The protein band labeled Pβ is present in the autoradiogram in large quantities in EDTA-lysozyme extracts obtained from the wild-type and *phoS*$^-$ strain grown and labeled in excess phosphate medium. Material at this position in the autoradiogram is absent in extracts obtained from cells grown and labeled in phosphate-limiting conditions. However, in the stained gel (Fig. 2a), band Pβ is present in EDTA-lysozyme extracts obtained from cells grown in excess phosphate or phosphate-limiting conditions. These results imply that the protein Pβ is a stable protein, which is synthesized only during growth in excess phosphate medium but which persists during phosphate-limited growth. Band Pα shows the same behavior in the autoradiogram (Fig. 2b), although it is only present in small amounts in the stained gel. Since the same protein samples are used for both gels, protein band Pα seems to be a minor periplasmic protein specifically labeled during growth in excess phosphate medium. Band Pγ is similar to bands Pα and Pβ in the autoradiogram but is present in the stained gel only in the *phoS*$^-$ mutant extracts. (This might be the result of a low protein concentration in the wild-type sample used here.) In Fig. 4, band Pγ can be seen in the stained sample of the wild-type strain grown in excess phosphate medium. Figure 4 also demonstrates that Pα and Pβ show the same pattern as in Fig. 2a. Protein bands Pα, Pβ, and Pγ are not solubilized by polymyxin sulfate treatment (Fig. 2a), and bands Pα and Pγ are not present in samples obtained by the osmotic shock procedure (Fig. 1).

Immunological detection of the presence of the *phoS* protein in four *phoR1c*$^-$ strains and the absence of the *phoS* protein in a deletion of the *phoB* region. The *phoR1c* mutant S3, analyzed in Fig. 4 and 5, is one of a series of independently isolated mutations mapping in the *phoR-phoB* region (16). Recently, an extended deletion that presumably includes the *phoR-phoB* sites has been isolated (5). We tested the other known *phoR1c*$^-$ strains and the

extended *phoR-phoB* deletion for the production of the *phoS* and *phoA* proteins by the immunological procedures described in Fig. 5. Figure 6 is a photograph of an Ouchterlony slide with periplasmic protein samples obtained by EDTA-lysozyme treatment of phosphate-starved cells as the antigens, and with antiserum to the *phoS* protein in the center well. In Fig. 6a it can be seen that the wild-type strain and the four *phoR1c*$^-$ strains produced the *phoS* antigen. Cross-reacting material was absent in the extract obtained from the *phoS*$^-$ mutant. In a separate slide (not shown) using antiserum to alkaline phosphatase in the center well, only the extracts obtained from the wild-type strain and the *phoS*$^-$ mutant contained the *phoA* antigen. In Fig. 6b we have tested extended deletion strain XPh24 of Brickman and Beckwith (5) and have compared it to its parent strain, XPh1a, a *phoR1a*$^-$ mutant. The parental strain XPh1a produced the *phoS* antigen after growth in excess phosphate medium (GP) or after phosphate starvation. The extended deletion strain showed no evidence of the *phoS* antigen in either medium. In a separate slide (not shown) using antiserum to alkaline phosphatase, the parental strain XPh1a synthesized alkaline phosphatase in either medium, whereas the deletion strain did not produce any detectable *phoA* antigen in either growth condition. Thus, all the *phoR1c*$^-$ strains tested produced the *phoS* protein, whereas they failed to produce any alkaline phosphatase. The extended deletion XPh24 failed to produce either *phoS* protein or alkaline phosphatase (the property of the *phoB*$^-$ point mutant LEP1 [Fig. 4 and 5]) and therefore should include the *phoB* region.

Production of the *phoS* protein in excess phosphate growth medium. Garen and Otsuji (10) reported that about 1 to 2% of the fully derepressed level of the R2a protein, now known to be the phosphate-binding protein, could be detected in wild-type cells during conditions of alkaline phosphatase repression. We have tested the periplasmic protein extracts obtained from three different wild-type strains by EDTA-lysozyme treatment for the presence of the *phoS* antigen after growth in excess phosphate media. In Fig. 7, it can be clearly seen that the extracts from both excess phosphate and phosphate-starved, wild-type cells contained the *phoS* antigen. Although accurate quantitation of the amount of *phoS* protein in these extracts was not done, it can be concluded that there is significant *phoS* protein synthesis during growth in excess organic phosphate medium (alkaline phosphatase repression conditions) and that this protein is located in the periplasmic space.

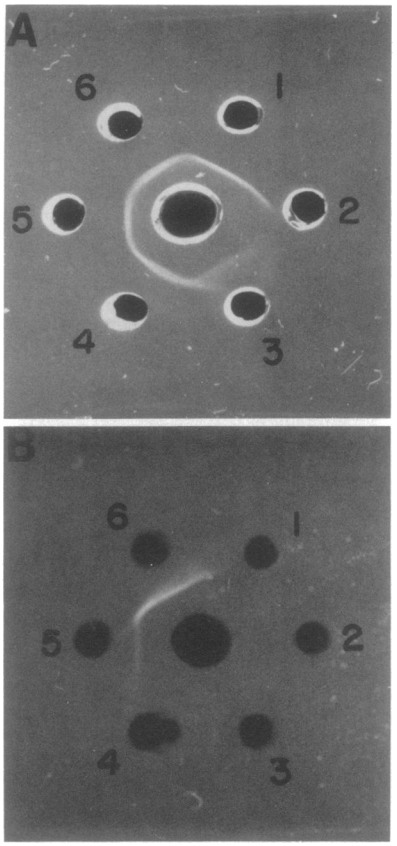

FIG. 6. *Immunological detection of the presence of the phoS protein in four phoR1c*$^-$ *strains and the absence of the phoS protein in a deletion of the phoB-phoR region. The antigens present in the outer wells were prepared by EDTA-lysozyme treatment. The center wells contained antibody to the phosphate-binding protein. The numbers in parentheses indicate the protein content of each well. (a) Periplasmic protein extracts from phosphate-starved cells: 1, GR5211 pst*$^+$ *pit*$^+$ *phoS*$^+$ *phoR*$^+$ *(3.5 μg); 2, GR5198 pst*$^+$ *pit*$^+$ *phoS*$^-$ *phoR*$^+$ *(2.3 μg); 3, S3 phoR1c*$^-$ *(5.0 μg); 4, G206 phoR1c*$^-$ *(6.2 μg); 5, H2 phoR1c*$^-$ *(6 μg); 6, H9 phoR1c*$^-$ *(7.5 μg). (b) Odd-numbered wells contained periplasmic protein extracts from cells grown in excess phosphate (GP) medium, whereas even-numbered wells contained periplasmic protein extracts from phosphate-starved cells. Because of the slow growth of the deletion strain in GP medium, samples in wells 1 and 2 were also obtained from cells that had been allowed to grow overnight in GP medium. XPh24-Δ(phoA phoB brnQ proC): 1 (3.0 μg), 2 (2.3 μg), 3 (1.3 μg), 4 (2.5 μg); XPh1a-(phoR1a*$^-$*): 5 (2.3 μg), 6 (2.0 μg).*

DISCUSSION

Periplasmic protein band P4 as described by Morris et al. (20) is a mixture of at least two proteins. We have identified band P4a as the phosphate-binding protein, product of the *phoS*

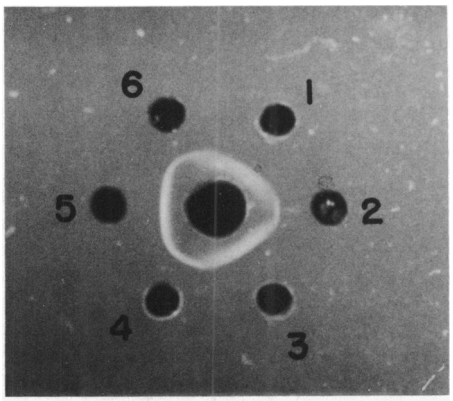

FIG. 7. *Immunological evidence that significant phoS protein is produced in wild-type strains during growth in excess organic phosphate (GP) medium. Antigens 1 and 2 were obtained by EDTA-lysozyme treatment; antigens 3 through 6 were obtained by osmotic shock treatment. Antibody against the phosphate-binding protein was present in the center well. Even-numbered wells contain periplasmic protein extracts obtained from cells grown in excess organic phosphate (GP) medium, whereas odd-numbered wells contain periplasmic protein extracts obtained from phosphate-starved cells. The numbers in parentheses indicate the protein content of each well. GR5154 pst$^+$ pit$^-$: 1 (32 μg), 2 (26 μg); GR5134 pst$^+$ pit$^+$: 3 (24 μg), 4 (24 μg); GR5211 pst$^+$ pit$^+$: 5 (3.5 μg), 6 (2.5 μg).*

gene. Previous identification of this protein on SDS-polyacrylamide gels was made difficult by the presence of another protein, band P4b, running at the same position, which is also controlled by the level of phosphate in the growth medium. We have been able to distinguish between these two proteins, since only band 4a is liberated by polymyxin sulfate treatment. Band 4a is absent in extracts of *phoS*$^-$ mutants, and in *phoS*$^+$ cells it cannot be radioactively labeled with [^{35}S]methionine. The synthesis of bands P4a and P4b is controlled by the level of phosphate in the growth medium and depends on the functioning of the positive control gene, *phoB*. In an accompanying paper (30), Yagil et al. show that purified phosphate-binding protein runs in the same position on SDS-acrylamide gels as band P4. In addition they observe alterations in the amount of band P4 protein in certain *phoS*$^-$ mutants.

In the course of these studies we have discovered the existence of a group of periplasmic proteins whose regulation is the reverse of that seen with alkaline phosphatase. Proteins Pα, Pβ, and Pγ are only synthesized during growth in excess phosphate medium and are not synthesized during growth in phosphate-limited medium. Pβ must be a stable protein since it can be detected by a protein-staining method in extracts prepared several hours after a shift to phosphate starvation conditions, but it cannot be radioactively labeled during limiting phosphate growth. Production of Pα and Pβ is independent of the known alkaline phosphatase control genes, since these proteins can be found in *phoB*$^-$ and *phoR1c*$^-$ cell extracts. Pβ is only produced in excess phosphate medium in the presence of the three alkaline phosphatase control mutations (*phoR1a*$^-$, *phoR1c*$^-$, and *phoB*$^-$), suggesting that its regulation is independent of any of the known alkaline phosphatase regulatory genes. The function of these proteins is not known, although it is tempting to speculate that they play a role in phosphate transport under high-phosphate conditions.

The function of most of the periplasmic proteins produced at maximum rate during growth in limiting P_i medium in unknown. We have previously hypothesized that the *phoS* protein imparts a degree of specificity to the PST phosphate transport system. *phoS*$^+$ cells appear to be able to discriminate against arsenate in the growth medium, since in the proper genetic background *phoS*$^+$ cells are arsenate resistant, whereas *phoS*$^-$ mutants in the same genetic background are arsenate sensitive (29). Since this specificity is also observed during growth in excess phosphate, we would predict that significant amounts of the *phoS* protein should be produced under these growth conditions. In the first report on the *phoS* protein, then called the R2a protein, Garen and Otsuji (10) reported that 1 to 2% of the fully derepressed levels of this protein could be detected immunologically in extracts of cells grown in high P_i medium (alkaline phosphatase repression conditions). In the present report we demonstrate that the *phoS* protein can be detected in the periplasmic fraction of cells grown in excess phosphate medium (conditions of alkaline phosphatase repression). However, *phoS* protein is produced at a maximum rate during growth in limiting phosphate medium. A possible function for this protein during phosphate limitation is suggested by our observation that the *phoS* protein is necessary for the rapid initial phase of phosphate accumulation into cells dependent upon the PST system for phosphate transport (manuscript in preparation).

In addition, *phoS*$^-$ mutants display alterations in the kinetic parameters of the PST P_i transport system. We have shown that the K_m and V_{max} are both increased in a *phoS*$^-$ mutant when compared with the *phoS*$^+$ parent strain (28). Kida has reported (15) altered P_i transport properties in several mutants presumed to be in the *phoS* cistron. However, certain of her strains (C93) produce normal amounts of the phosphate-binding protein antigen and must

not be mutants in *phoS*. These strains might contain a *phoT*⁻ or *pst*⁻ mutation. Two of her mutants, C75 and KK26, do lack the phosphate-binding protein and show slightly decreased growth rates in limiting phosphate medium and small alterations in P_i transport parameters. In agreement with Kida, we have now determined that some of the *phoS*⁻ strains used in our previous study (28) can give rise to derivatives, showing decreased growth rates in limiting phosphate medium and reduced arsenate sensitivity, when introduced into certain genetic backgrounds. The basis of this variability is not yet understood.

It is possible that some of the other periplasmic proteins produced during growth in limiting phosphate medium may be involved with other P_i transport systems. However, at this time we have not observed any changes in the activity of the other constitutive phosphate transport system (PIT) in the absence of the other periplasmic proteins (*phoB*⁻, *pst*⁻ cells; manuscript in preparation).

The ability to specifically identify the *phoS* protein in polyacrylamide gels has led to the discovery that the regulation of *phoA* and *phoS* synthesis is not identical. Expression of both genes, although widely separated on the *E. coli* genome, is dependent on the *phoB*-positive control gene. No detectable *phoA* or *phoS* products are found in *phoB*⁻ strains grown under limiting or excess phosphate conditions. (The other periplasmic proteins controlled by the level of P_i in the medium are also not synthesized in a *phoB*⁻ strain [P2, P4b].) However, there is a difference in *phoA* and *phoS* synthesis in strains carrying *phoR1c*⁻ mutations; *phoA* protein is not produced, whereas the *phoS* product is present in normal amounts. This result has been obtained both by examining the protein band profile on polyacrylamide gels (*phoR1c*⁻ [*S3*]) and by immunological techniques with specific antisera (all *phoR1c*⁻). Thus, we confirm the report of Garen and Otsuji (10) that fully derepressed amounts of the *phoS* protein are present in *phoR1c*⁻ mutants.

The recent discovery of complementation between *phoR1a* and *phoR1c*⁻ mutations (16) suggests that these mutations occur in separate cistrons and specify two different polypeptide chains (the possibility of intracistronic complementation has not been ruled out). Although *phoB*⁻ mutations were shown to complement with *phoR1a* mutations (4), *cis-trans* tests have not been performed with *phoB*⁻ and *phoR1c*⁻ mutations. Thus, there are probably two or three cistrons in the *phoB-phoR* region involved in the regulation of alkaline phosphatase and the other periplasmic proteins whose synthesis depends upon the phosphate concentration in the growth medium. The *phoB* product is required for both *phoA* and *phoS* synthesis (and band P2 synthesis); it seems to play a positive role in the control of these proteins. The *phoR1c* product is required for *phoA* synthesis but does not seem to be required for *phoS* synthesis. If the *phoR1c* product is to have a general positive regulatory effect on the proteins whose synthesis depends on phosphate levels in the growth medium, then it is possible that the four *phoR1c*⁻ mutations studied here result in the destruction of the function required for *phoA* synthesis but that the function required to activate *phoS* synthesis is retained. Consistent with this hypothesis is the fact that none of the existing *phoR1c*⁻ strains contain an amber mutation (A. M. Torriani, personal communication). The *phoR1a-phoR1b* product seems to have a negative effect, since mutations in this cistron lead to constitutive synthesis of both *phoA* and *phoS* (8). A detailed model for the control of alkaline phosphatase synthesis and the synthesis of the other phosphate-controlled periplasmic proteins has been proposed (20). This model needs to be revised to include the findings of this paper. We will, however, postpone the formulation of a more detailed model until further genetic and in vitro regulation studies are performed.

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