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# Pleiotropic Effects of Mutations Involved in the Regulation of *Escherichia coli* K-12 Alkaline Phosphatase

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Induction of alkaline phosphatase in wild-type *Escherichia coli* K-12 leads to the appearance of three new proteins in addition to alkaline phosphatase in the periplasmic space of the bacteria. These proteins are detected in autoradiograms of sodium dodecyl sulfate-acrylamide gel electropherograms of extracts from cells labeled with [$^{35}$S]methionine. Studies with constitutive mutants defective in the three genes *phoS*, *phoT*, and *phoR* that have been shown to regulate alkaline phosphatase synthesis indicate that the three periplasmic proteins are co-regulated with alkaline phosphatase. A mutant that has a deletion in the alkaline phosphatase structural gene *phoA* produces the three proteins, but a newly discovered mutant *phoB* that has a defect in the expression of alkaline phosphatase fails to produce the three proteins. *phoB* mutants are shown here to be unable to make detectable amounts of alkaline phosphatase polypeptides, as measured by immunoprecipitins or acrylamide gel electropherograms. On the basis of these results we suggest a new model for the regulation of alkaline phosphatase biosynthesis. In this model, a ternary complex composed of *phoB*$^+$ and *phoR*$^+$ gene products and an internal metabolite functions as a positive control element to regulate the transcription of several cistrons coding for periplasmic proteins.

Synthesis of alkaline phosphatase (EC 3.1.3.1) in *Escherichia coli* K-12 is controlled by the action of at least four genes denoted *phoS*, *phoT*, *phoR*, and *phoB* (2, 4, 24). (In the first report of this mutant [2], it was noted as *phoT*.) All of these genes map at sites distinct from the *phoA* alkaline phosphatase structural gene (Fig. 1). In wild-type *E. coli* K-12, alkaline phosphatase is induced only when the level of inorganic phosphate ($P_i$) in the culture medium becomes limiting for growth (20), but *phoS*, *phoT*, and *phoR* mutants produce the enzymes constitutively.

Recently, Willsky et al. showed that *phoT* affects the transport and accumulation of $P_i$ and suggested that the *phoS* gene product is also involved in $P_i$ transport (24); thus, defects in either of these genes would influence the internal level of $P_i$ in much the same way as a depletion of $P_i$ from the growth medium affects wild-type cells. The *phoR*$^+$ product was proposed to function in both repression and induction because most *phoR* mutants were unable to make maximal amounts of enzyme even after growth limitation for $P_i$ (6, 7).

The *phoB* gene was discovered when a new class of phenotypically alkaline phosphatase-negative mutants ($P^-$) were analyzed by genetic mapping (2). These $P^-$ mutants mapped at a site close to but distinct from the *phoR* gene and from the *phoA* structural gene. *phoB*$^+$ was

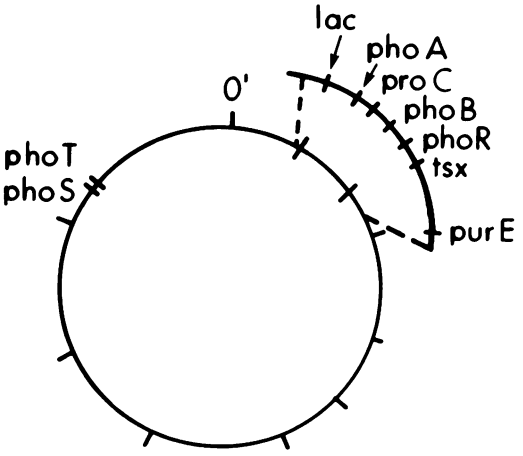


FIG. 1. *Genetic map (Taylor) of E. coli genes involved in alkaline phosphatase formation. Based on material presented in references 2 and 24.*

dominant in merodiploids and *phoB* was epistatic to the other three regulatory genes, i.e., a *phoB phoR* double mutant was phenotypically $P^-$ (2). The function of the $phoB^+$ gene product has not been determined. One hypothesis that could account for a *phoB* phenotype is that this gene codes for a protein that is essential for either the transport or assembly of alkaline phosphatase subunits in the periplasmic space of the bacterial cell—the region where this enzyme becomes localized (10, 14). We tested this model by looking in extracts of *phoB* cells for polypeptides that were antigenically related to alkaline phosphatase subunits or nascent polypeptide chains and by examining in high-resolution sodium dodecyl sulfate (SDS)-polyacrylamide slab gels the pattern of proteins that become labeled under conditions of alkaline phosphatase derepression. Our results indicate that *phoB* is not involved in the transport or maturation of alkaline phosphatase. However, from the latter experiments, we discovered that *phoB* mutants were lacking three additional proteins as well as alkaline phosphatase, and all of them were localized in the periplasmic space of the wild-type cell. Further studies revealed that these periplasmic proteins were coregulated by those genes controlling induction of alkaline phosphatase. On the basis of these experiments, we have proposed a new model for the regulation of *E. coli* alkaline phosphatase.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains used in these experiments are listed in Table 1; all are derived from *E. coli* strain K-12.

All cultures were grown in a tris(hydroxymethyl)-aminomethane (Tris)-salts minimal medium, previously described (16). When required, amino acids and adenine were added at 20 μg/ml. To derepress cells for formation of alkaline phosphatase, exponentially growing cultures were harvested when they reached a density of about $5 \times 10^8$ cells per ml and were resuspended in medium lacking $P_i$. After approximately 2 h at 37 C, cultures were harvested. The level of alkaline phosphatase in K-12 strains reaches 75% of the fully derepressed levels under these conditions.

Strain RTE2, a revertant of strain LEP1, was isolated after mutagenesis with ethylmethane sulphonate (EMS). LEP1 cells ($2 \times 10^9$) were plated on a Tris-salts minimal medium containing adenine, proline, tryptophan, and β-glycerolphosphate ($10^{-4}$ M) as the source of phosphorous. A drop of EMS was placed in the center of the plate and cells incubated at 37 C. Within 2 to 3 days a number of colonies appeared. After replating, the colonies were further selected for alkaline phosphatase production by spraying with *p*-nitrophenylphosphate (10 mg/ml in M Tris-chloride, pH 8). Revertant colonies turned yellow quickly. One of those yielding intense color was picked for further analysis.

**Labeling of cell proteins and preparations of subcellular fractions.** [$^{35}$S]methionine (5 μCi, specific activity of 118 Ci/mM from Amersham-Searle) was added to 25 ml of either exponentially growing cultures or cells grown for 2 h in $P_i$-free medium. Fifteen minutes after addition of the isotope, cells were harvested at 4 C was washed with cold buffer containing 10 mM Tris-chloride, pH 7.4, 1 mM $MgCl_2$, 10 μM $ZnCl_2$. To prepare whole cell extracts the pellet of washed cells was suspended in 0.2 ml of 2% SDS and sonically disrupted. Extracts were heated at 75 C for 30 min prior to analysis. To prepare periplasmic fractions, harvested cells were suspended in 0.4 ml of a solution containing 25% sucrose, 10 mM Tris-chloride, pH 8.0. Lysozyme (50 μliters of 5 mg of protein per ml) and ethylenediamine tetraacetic acid (50 μliters of 20 mM, pH 8.0) were added and the cells incubated for 15 min at 23 C. Formation of spheroplasts was monitored by measuring $A_{540}$ of a 1:100 dilution of the cells; we routinely worked with preparations in which greater than 90% of the cells were converted to spheroplasts. The treated cells were centrifuged at 4 C and the supernatant fraction is referred to as the periplasmic fraction. Prior to analysis in polyacrylamide gel electrophoresis, a sample was brought to a final concentration of 2% SDS and heated at 75 C for 30 min.

TABLE 1. *Properties of strains*

| Designation | *pho* Genotype | Phenotype for alkaline phosphatase | Genotype for other markers | Source |
|---|---|---|---|---|
| K10 | Wild type | $P^+$, inducible | + | 5, 13 |
| E15 | *phoA* | $P^-$ (deletion) | + | 5, 13 |
| C2 | *phoR* | $P^-$, constitutive | + | 4 |
| C84 | *phoT* (am)[a] | $P^-$, constitutive | + | 8, 9 |
| C86 | *phoS*[a] | $P^-$, constitutive | + | 8, 9 |
| D1 | *phoR* | $P^-$, constitutive | + | E. Yagil |
| D2 | *phoR* | $P^-$, constitutive | + | E. Yagil |
| J1 | *phoR* | $P^-$, constitutive | + | E. Yagil |
| LEP1 | *PhoB*[b] | $P^-$ | *pro*$^-$, *trp*$^-$, *ade*$^-$ | 2 |
| RTE2 | *phoBphoR* | $P^-$, inducible | *pro*$^-$, *trp*$^-$, *ade*$^-$ | This work |

[a] Originally noted *phoR2*.
[b] Originally noted *phoT*.

The pellet from the lysozyme-treated cells was resuspended in 0.5 ml of 2% SDS and sonically disrupted; this sample is referred to as a spheroplast fraction. In cases where membrane fractions were analyzed, the pellet of spheroplasts was resuspended in 4 ml of buffer containing 400 $\mu$g of deoxyribonuclease, 100 $\mu$g of ribonuclease, 10 mM mercaptoethanol, and 50 mM Tris-chloride, pH 7.4. This solution was frozen in dry-ice acetone and thawed to 23 C. After three cycles of freezing-thawing, the spheroplasts were centrifuged at 4 C for 30 min at 27,000 $\times$ $g$. The supernatant fraction is termed the cytoplasmic fraction. The pellet was suspended in 4 ml of a buffer that contained 10 mM Tris-chloride, pH 7.4, 1.5 mM NaCl (RSB). This solution was added to the top of a discontinuous gradient of sucrose solution made by first adding 4 ml of 1.8 M sucrose in RSB, and overlaying this with 3 ml of 0.5 M sucrose in RSB. After centrifugation for 12 to 16 h in a SB 283 rotor in the International Equipment Co. B-60 ultracentrifuge at 30,000 rpm, a 2-ml fraction was carefully removed from the interface layers of the sucrose solution. This fraction has been shown to contain >80% of the cell membranes (3); it was subsequently centrifuged for 1 h at 45,000 rpm in a 65 Spinco rotor in the model L ultracentrifuge. The pellet was resuspended in 0.5 ml of 2% SDS, sonically disrupted, and heated at 75 C for 30 min. This fraction is termed the membrane.

**Analysis of proteins by acylamide gel electrophoresis.** Samples (5 to 20 $\mu$liters) were layered in slots formed in thin (2 mm) slabs composed of a lower gel of 10% acrylamide in 0.2 M Tris-chloride, pH 8.8, and 0.1% SDS, and an upper spacer gel of 5% acrylamide in 75 mM Tris-chloride, pH 6.8, 0.1% SDS as described by Laemmli (12). The apparatus for performing this kind of electrophoresis has been recently described (22). Electrophoresis was performed at 23 C at 80 V (constant) for 3.5 h. The slab was transferred to Whatman 3 MM paper, dried while subjected to vacuum, and the dried gel was exposed to Kodak No Screen X-ray film. Exposure times varied from 5 to 14 days, depending on the amount of radioactive proteins in the gels. The amount of labeled protein in a sample added to the gel ranged from 5,000 to 20,000 counts/min.

**Analysis of proteins by precipitation with antibodies.** The antibodies used in these experiments and the methodology employed has been described in detail elsewhere (3, 17). The preparation of antibodies forms a precipitate with inactive alkaline phosphatase dimers and denatured subunits but does not cross-react with the active enzyme.

**Assay for enzymatic activity and radioactivity.** The procedure for measuring alkaline phosphatase activity by using $p$-nitrophenylphosphate has been described (18). [$^{35}$S]methionine-labeled protein was counted as described (1).

**Materials.** All chemicals were reagent grade. $p$-Nitrophenylphosphate and DNase were from Sigma Chemical Co., St. Louis. Acrylamide was recrystallized prior to use in electrophoresis.

## RESULTS

**Pattern of proteins noted in cultures derepressed for alkaline phosphatase.** The technique of acrylamide gel electrophoresis in buffers containing SDS together with an autoradiographic detection procedure has afforded a method for examining protein species to a high degree of resolution (12, 22). We have utilized some of the recently developed procedures to examine the pattern of proteins from subcellular fractions of several *E. coli* strains grown under conditions that affect alkaline phosphatase formation. A typical set of results are shown in Fig. 2 which depicts the protein bands localized to the cell cytoplasm, membrane, and periplasm. These fractions were obtained from exponential and $P_i$-starved cultures of wild-type K10. Only in the periplasmic fraction (band C−, Fig. 2) are there significant differences in the protein pattern; four new bands, noted P-1, P-2, P-3, and P-4, appear or increase in intensity as a result of growth limitation in a $P_i$-free medium. Band P-1 is the alkaline phosphatase subunit of molecular weight 43,000. This has been established by precipitating the periplasmic fraction with a preparation of antibodies specific to active alkaline phosphatase (17) and subjecting the antibody-antigen precipitate —after dissolving in SDS—to electrophoresis in the same SDS gel. Only band P-1 was observed. As noted below, band P1 is absent in an *E. coli* mutant containing a deletion in the alkaline phosphatase structural gene. Protein band P-2 may be formed in small amounts during exponential growth of the cells but its synthesis is greatly increased after cells are starved for $P_i$.

We have estimated the molecular weights of P-2, P-3, and P-4 to be 40,000, 35,000, and 30,000, respectively, on the basis of a comparison of their mobilities in SDS gel electrophoresis with the mobilities of alkaline phosphatase subunit (molecular weight = 43,000), the heavy chain of $\gamma$-globulin (molecular weight = 50,000), and the light chain of $\gamma$-globulin (molecular weight = 21,000). When we analyzed the pattern of periplasmic proteins of $P_i$-starved cells that were fractionated on a G-100 Sephadex column (data not presented), we observed that with the exception of alkaline phosphatase which eluted near to void volume of the column, other proteins formed in $P_i$-starvation eluted in a molecular weight range estimated to be between 30,000 and 50,000. In its native active state alkaline phosphatase is a dimer of molecular weight 86,000, but we would conclude that the three other proteins noted above have a native molecular weight equivalent to that observed in SDS gels, i.e., they are monomeric in their native conformation.

**Effect of mutations in phoS, phoT, and phoR on periplasmic proteins.** *phoS* and *phoT* mutants are altered in their ability to accumu-

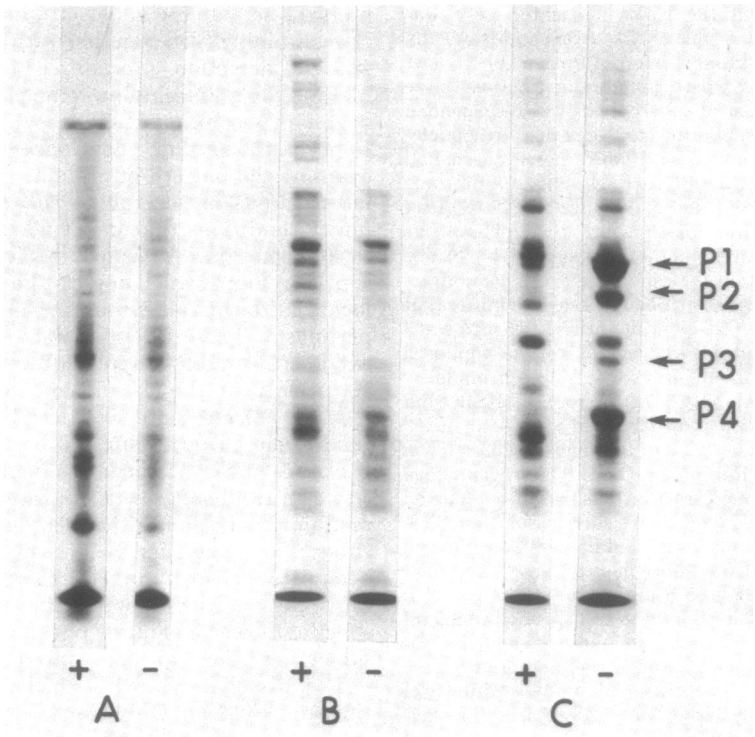


FIG. 2. *Pattern of proteins obtained from subcellular fractions of strain K10 grown exponentially or starved for $P_i$. Set A was from membranes; set B was from cytoplasm; set C was from periplasm. (+) Refers to the extracts from exponentially growing cells; (−) refers to extracts from cells labeled after 2 h of starvation for $P_i$. The amount of labeled protein (counts per minute) added was A+, 7,200; A−, 4,000; B+, 8,000; B−, 5,000; C+, 5,000; C−, 11,000.*

late $P_i$ in the bacterial cytoplasm (24) and these mutants are also constitutive for formation of alkaline phosphatase. The patterns of periplasmic proteins from the *phoS* mutant C86 and the *phoT* mutant C84 are shown in Fig. 3. Extracts were obtained from cells growing both exponentially and limited for $P_i$. Bands P-1, P-2, and P-4 are clearly present under both growth conditions in the mutants; band P-3 appears in significant amounts only in extracts from $P_i$-starved cells. Thus, *phoS* and *phoT* mutations are pleiotropic for regulating the formation of at least three periplasmic proteins.

The control of alkaline phosphatase production by the *phoR* gene product is complex; most *phoR* mutants make low amounts of enzyme (about 20% the fully derepressed level) during exponential growth of the mutant, and this level does not increase during starvation for $P_i$ (Table 2, strains D1 and J1). A few *phoR* mutants show an increased level of enzyme after starvation for $P_i$ (D2, Table 2) and a more rare type of *phoR* mutation does not make enzyme under any growth condition (7). The pattern of periplasmic proteins from three *phoR* mutants reveals that the P-1, P-2, and P-4 protein (P-3 was not resolved in the gels) are formed constitutively and in amounts roughly equivalent to the levels of alkaline phosphatase made in these constitutive mutants (Fig. 4A, B, and C); strains D1 and D2 made about the same amount of enzyme and J1 made twice as much (Table 2). The pattern from K10 (column D, Fig. 4) was from a culture starved for $P_i$ and there were two and one-half times more labeled protein added to the gel than that used for the *phoR* strains. For this reason, one cannot compare quantitatively the K10 pattern to the others. The results noted in Fig. 4 indicate that the *phoR* gene product exerts a pleiotropic effect on three periplasmic proteins.

**Effect of mutations in the phoA gene on periplasmic proteins.** A variety of mutants with defects in the alkaline phosphatase structural gene have been isolated. They include a deletion mutant, point mutants that make immunologically cross-reacting protein (CRM), and nonsense mutants that make polypeptide

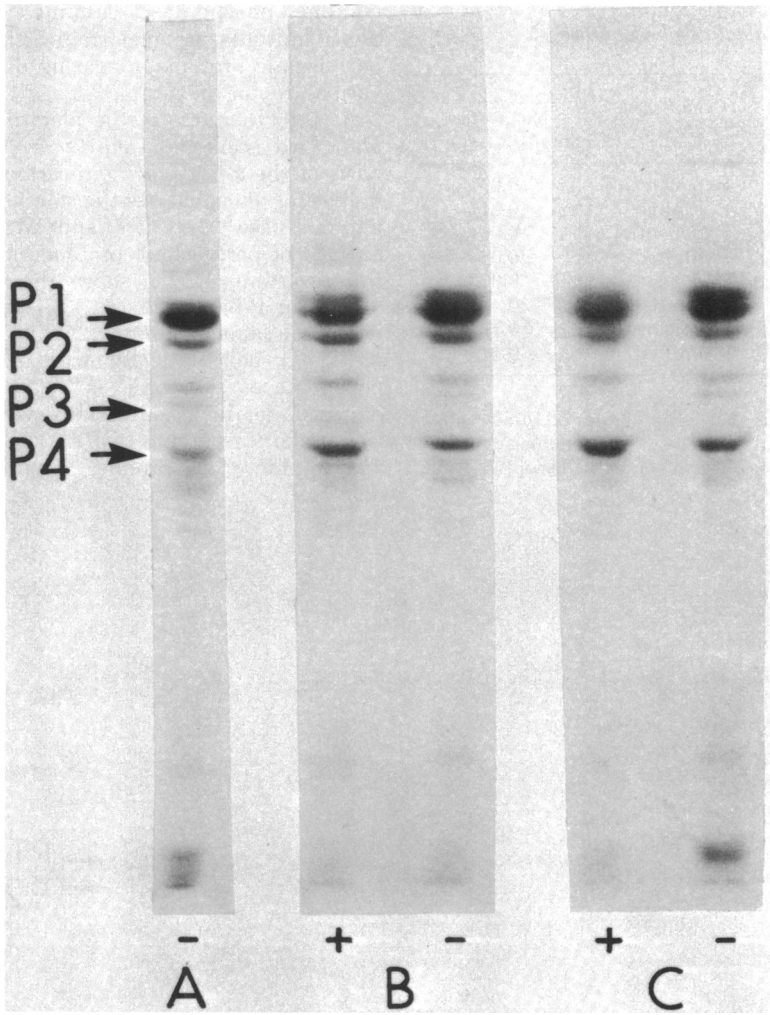


FIG. 3. *Pattern of periplasmic proteins from phoS and phoT mutants. Set A was from K10 starved for $P_i$; set B was from phoT; set C was from phoS. (+) Refers to extracts from exponentially growing cells; (-) refers to extracts from $P_i$-starved cell. The amount of labeled protein was the same in all samples (4,000 counts/min).*

fragments (5, 13, 19). The pattern of periplasmic proteins from the deletion mutant E15 shows that protein band P-1 is absent, as expected, but P-2 and P-4 are present (Fig. 5). The band P-3 is detected in lower amounts. A similar result was observed with extracts from a *phoA* nonsense mutant reported to have a lesion near the amino-terminal part of the gene (19). A point mutant that makes a CRM displayed a pattern identical to that of *phoA*$^-$ strains. Thus, induction of proteins P-2 and P-4 is independent of the formation of complete, functional alkaline phosphatase. Band P-3 behaves anomalously and we are uncertain what factors control its formation.

**Effect of mutations in the phoB gene on periplasmic proteins.** Strains of *phoB* are unable to make active alkaline phosphatase—they are phenotypically P$^-$; yet *phoB* mutations lie outside the *phA* structural gene (2). To determine if *phoB* mutants make inactive forms of alkaline phosphatase, we examined the cytoplasmic and periplasmic fractions of strain LEP1 in the SDS gel system. When compared with extracts of K10, those from *phoB* are missing the P-1, P-2, and P-4 bands (Fig. 6). The periplasmic extract from exponentially grown LEP1 (Fig. 6A+) was identical to that of K10 (Fig. 2C+); only under $P_i$-starved conditions was LEP1 distinguishable from K10 (Fig. 6A- and B). It appears that *phoB* strains are unable to make a polypeptide related to the

TABLE 2. *Effect of phoR mutations on formation of alkaline phosphatase*

| Strain | Condition of growth | Act of alkaline phosphatase (units/$A_{540}$ cells)[a] |
|---|---|---|
| K10 | Exponential | 0.007 |
| | $P_i$-limited | 0.31 |
| D1 | Exponential | 0.06 |
| | $P_i$-limited | 0.08 |
| D2 | Exponential | 0.07 |
| | $P_i$-limited | 0.43 |
| J1 | Exponential | 0.17 |
| | $P_i$-limited | 0.14 |

[a] One unit is one micromole of *p*-nitrophenol formed per minute at 37 C in M Tris-chloride, pH 8.0 (18). A sample of cells was sonically disrupted prior to assay.

alkaline phosphatase subunit. In addition, *phoB* mutants are pleiotropic; the three new periplasmic proteins appearing during $P_i$ starvation are not detectable.

As a further test of the pleiotropic effect of *phoB*, we isolated a *phoB*$^+$ revertant on the basis of the ability of the revertant to grow on $\beta$-glycerol-phosphate as the sole source of phosphorous (see Materials and Methods). The pattern of periplasmic proteins obtained from this revertant (RTE2) shows the reappearance of all four P-bands (Fig. 6C). Strain RTE2 produced the same amount of alkaline phosphatase, measured enzymatically with *p*-nitrophenyl-phosphate as substrate, as that made in K10 grown under identical conditions of $P_i$ limitation. Like its parent, LEP1, strain RTE2 was auxotrophic for proline, tryptophan, and adenine.

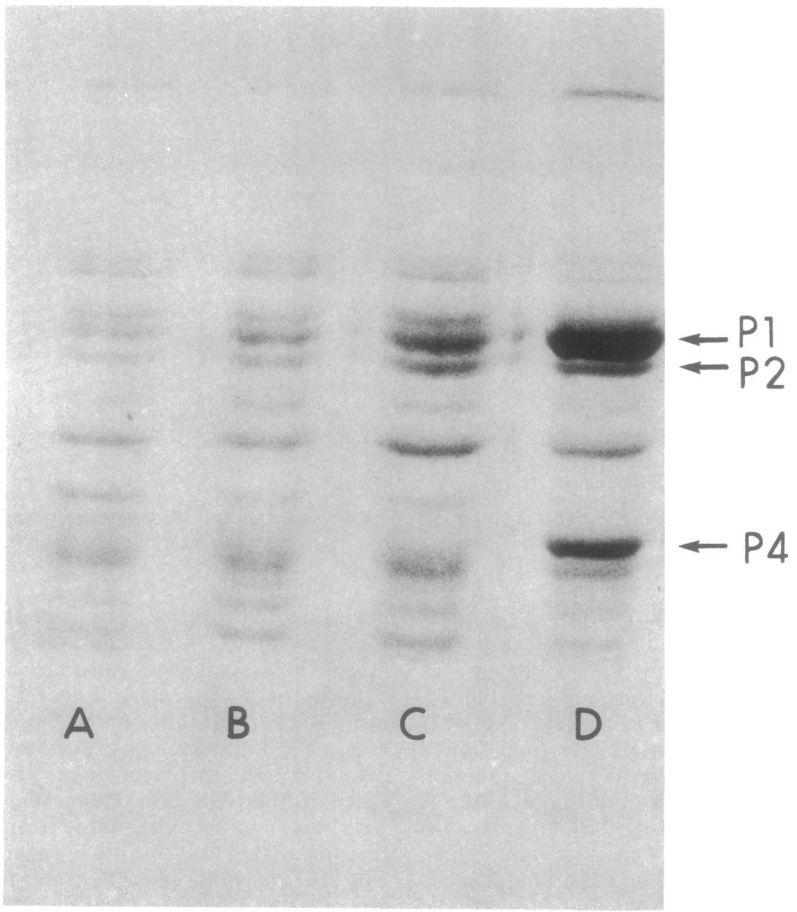


FIG. 4. *Pattern of periplasmic proteins from three different phoR mutants. All samples except D were from cells grown exponentially; D was from $P_i$-starved cells. (A) Was from D1; (B) was from D2; (C) was from J1; (D) was from K10. See Table 2 for amounts of alkaline phosphatase (P-1 band) in the extracts. Band P-3 was not resolved in this electropherogram. The amount of labeled protein (counts per minute) added was A, 3,700; B, 3,400; C, 4,300; D, 8,000.*

We have also examined the LEP1 periplasmic fraction in polyacrylamide gels containing ampholytes with a pH range between 4 and 8 (an isoelectric focusing system) as well as in polyacrylamide gels containing Tris-chloride buffer, pH 8.8, but no SDS. In both cases, a comparison of periplasmic proteins from the LEP1 strain with those formed by strain K10 showed an absence of alkaline phosphatase and three other bands from the LEP1 strain. In the isoelectric focusing system, these P-proteins appear in the pH range of 4.5 in comparison to alkaline phosphatase which is detected at pH 6.0.

There was the possibility that a *phoB*$^+$ gene product was required for transporting alkaline phosphatase polypeptides as well as other periplasmic proteins from their cytoplasmic site of synthesis to the periplasm. If this were the case, we should expect to find alkaline phosphatase subunits in the cell cytoplasm. Previous studies have shown that only a very minor fraction of alkaline phosphatase is detectable in the bacterial cell cytoplasm of K10 (3, 21). An SDS gel electropherogram did not show any extra amount of protein in the region expected for alkaline phosphatase (see above), but to confirm that there were no *phoA* gene products in the cytoplasm of *phoB* strains we examined cytoplasmic extracts of LEP1 for protein that could be precipitated with antibodies specific for alkaline phosphatase polypeptides. These antibodies are able to cross-react with inactive protein as well as subunits of the protein and nascent polypeptide chains (3). The results of these experiments are reported in Table 3. No evidence was obtained for alkaline phosphatase-like polypeptides in LEP1 cytoplasm; the amount of antigenic material in the cytoplasm was no greater than that detected in the E15 mutant which has a deletion in alkaline phosphatase structural gene or in the wild-type K10 strain. The periplasmic fraction of strain K10 shows the presence of large amounts of alkaline phosphatase. These results lead us to propose that the *phoB* gene product is an essential positive control element for the synthesis of alkaline phosphatase polypeptides.

## DISCUSSION

The work described here shows that there are at least four proteins in the *E. coli* K-12 periplasm that are synthesized under the same conditions as those previously established for the formation of *E. coli* alkaline phosphatase. Mutants that are constitutive for alkaline phosphatase are also constitutive for at least two of the three new proteins and a mutant (*phoB*) phenotypically negative for alkaline phosphatase fails to make these proteins.

These data help to explain our previous results describing increases in the amount of proteins in K-12 periplasmic fractions after starvation cultures for $P_i$ (15). During exponential growth, about 5 to 7% of the cell protein can be found in the periplasmic fraction but this value increases to 15 to 20% upon starvation for $P_i$. Only one-third of the increase could be attributed to the alkaline phosphatase. We would now propose that the balance of the periplasmic fraction is composed primarily of the P-2 and P-4 proteins discovered in the

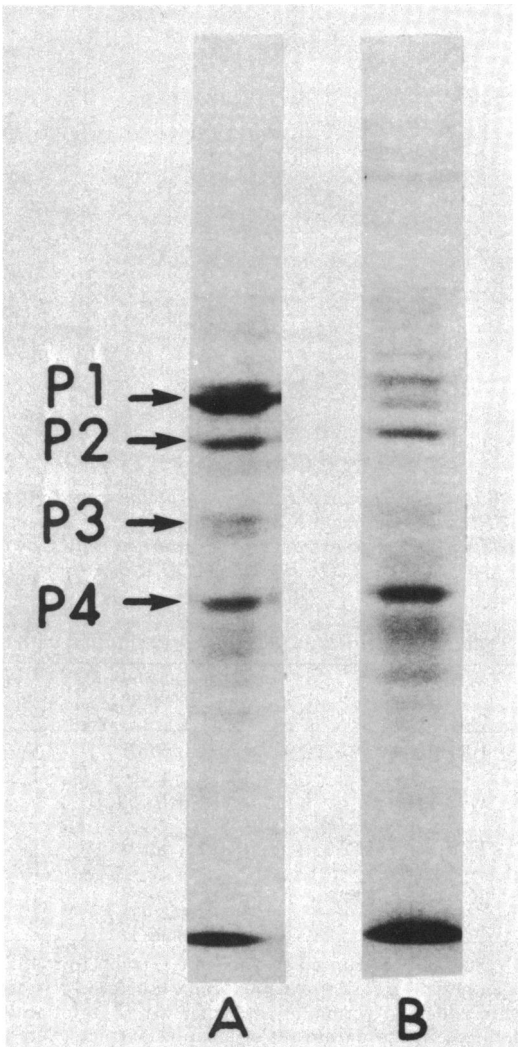


FIG. 5. *Pattern of periplasmic proteins from phoA*$^+$ *and phoA (deletion) mutant. Both cultures were labeled after starvation for* $P_i$. *(A)-wt K10, 8,600 counts/min; (B)-E15, 5,600 counts/min.*

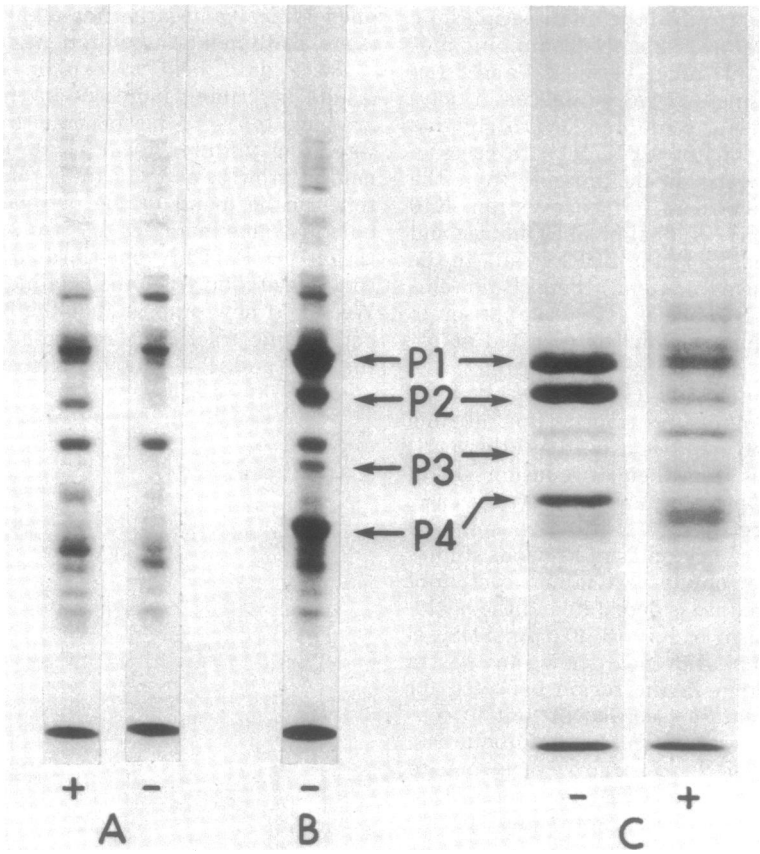


FIG. 6. *Pattern of periplasmic proteins from K10, phoB, and phoB⁺ (revertant). Set A was from phoB strain LEP1; set B was from K10; set C was from phoB⁺ revertant. (+) Refers to extracts from exponentially growing cells, (−) refers to extract from $P_i$-starved cells. The amount of labeled protein (counts per minute) added was A+, 4,200; A−, 3,200; B−, 11,000; C−, 6,800; C+, 4,800.*

TABLE 3. *Quantitative immuno-precipitation with cytoplasmic extracts of phoB and phoA E. coli strains*[a]

| Strain | Genotype | Amt added (counts/min) | Amt in precipitin (counts/min) | Percent in precipitin |
|---|---|---|---|---|
| LEP1 | *phoA*⁺, *phoB* | 32,000 | 231 | 0.7 |
| | | 82,000 | 359 | 0.4 |
| E15 | *phoA*(del), *phoB*⁺ | 60,000 | 236 | 0.4 |
| | | 90,000 | 497 | 0.5 |
| K10 | *phoA*⁺, *phoB*⁺ | 34,000 | 188 | 0.5 |
| | | 68,000 | 287 | 0.4 |
| K10 (periplasmic fraction) | *phoA*⁺, *phoB*⁺ | 6,500 | 2,400 | 37 |
| | | 11,500 | 3,870 | 34 |

[a] Sonically disrupted cytoplasmic extracts in 2% SDS were from preparations of spheroplasts of cells that had been grown in $P_i$-limited medium 2 h before [$^{35}$S]methionine was added. Samples of extract (10 to 20 μliters) were added to antibodies, together with 5 μg of antigen, to obtain specific co-precipitins (see reference 3 for details). In a separate set of samples extracts were added and antigen was omitted, but no differences were obtained between samples with antigen and those without in the percent protein precipitated. The periplasmic fraction of K10 (last line of table) was heated in 2% SDS at 75 C for 30 min in order to convert the alkaline phosphatase to subunits, and no antigen was included in the antibody precipitation reaction.

present work. The function of these proteins is unknown, but they might be expected to be involved in phosphate metabolism in bacteria. It is unlikely that either of them is related to a repressor protein studied by Garen (7). Mutant C86 which is shown here to make all four "P" bands makes less than 10% of the amount of this repressor protein as does wild type (7). The mutation in C86 may, in fact, be a nonsense mutation because the immunological tests for the putative repressor protein in C86 showed a value for a cross-reacting protein (CRM) to be only 6% that of wild-type strains (7). In addition, the *phoT* strain (C84) utilized here contains an amber mutation and would not be expected to make complete polypeptides. Extracts of these mutants had all four protein bands and, on this basis, we do not believe that either of the bands is a product of the *phoS*$^+$ or *phoT*$^+$ genes.

The results presented here together with other recently reported data about alkaline phosphatase regulation require that the established model for control of alkaline phosphatase formation be modified. In the original model devised by Garen (7), the *phoR*$^+$ gene product was postulated to function differently from the *lac* repressor which is the *I*$^+$ gene product. The major difference between these two regulator genes was that most *I*$^-$ mutants synthesized more β-galactosidase than did induced *I*$^+$ cells (11), whereas most *phoR* mutants made lower amounts of alkaline phosphatase, even during induction. For this reason, the *phoR* product was thought to be an inducer that reacted with $P_i$ in the presence of an enzyme coded for by the *phoR2*$^+$ gene (the *phoS*$^+$, *phoT*$^+$ complex) to form a repressor (7). In formulating a new model, the following data must be considered. (i) *phoS*$^+$ and *phoT*$^+$ are dominant and involved in the transport and accumulation of $P_i$ inside the cell. (ii) *phoR*$^+$ is trans-dominant (6) and *phoR* mutants are epistatic to *phoS* and *phoT*; most *phoR* mutants are unable to respond to further effects caused by starvation of cells for $P_i$. (iii) *phoB*$^+$ is trans-dominant.

Thus, both *phoR*$^-$ and *phoB*$^-$ gene products play critical roles in regulating alkaline phosphatase synthesis. The *phoR*$^+$ gene product is necessary for repression as well as for full expression of the *phoA*$^+$ structural gene (7). Data presented in Table 2 confirm this previously established observation and extend control by the *phoR* gene to production of three other periplasmic proteins. The *phoB*$^+$ gene product has been shown here not to be involved in the maturation of inactive alkaline phosphatase polypeptides and it most likely acts at the site of transcription of the mRNA. There are no explicit data showing that regulation of alkaline phosphatase synthesis is at the level of transcription rather than at the level of translation. However, alkaline phosphatase messenger ribonucleic acid has a half-life of 2 to 2.5 min (25). This time is similar to other systems in *E. coli* where regulation has been found to be expressed at the level of transcription, and in the following model we have assumed that regulation occurs at transcription.

The model we propose (Fig. 7) states that the *phoB* gene product is a protein "B" with binding sites for a promoter region of the *phoA* cistron and for the *phoR* gene product "R". The R protein, in addition to binding B, has a binding site for an internal metabolite "x" which accumulates when the level of $P_i$ in the cell cytoplasm drops below a critical value. This metabolite is not $P_i$ per se; Wilkins has shown that alkaline phosphatase can be induced under conditions that do not change the internal level of $P_i$ (23). In the absence of "x", all of "B" is in the complex B-R and this complex cannot bind effectively to *phoA* promoter. Accumulation of "x" as a result of mutations in *phoS* or *phoT* or starvation of cells for $P_i$ leads to a B-R-x complex which can bind to a *phoA* promoter. Finally, "B" itself can bind to *phoA* promoter but not as effectively as the B-R-x complex.

This scheme is not a unique one but it is consistent with all the data so far reported on alkaline phosphatase regulation. *phoB* mutants make defective B proteins and these cannot interact with *phoA* promoters. Most *phoR* mutants will make completely defective R proteins thereby allowing transcription only from a B-promoter interaction ($K_1$ in Fig. 7); a few *phoR* mutants (D2 in Table 2) will make partially defective R proteins whose activities may be "rescued" by interaction with the B protein and go on to yield a functional B-R-x complex when the cells starve for $P_i$. A more rare *phoR* mutant may make an R protein that could bind all B

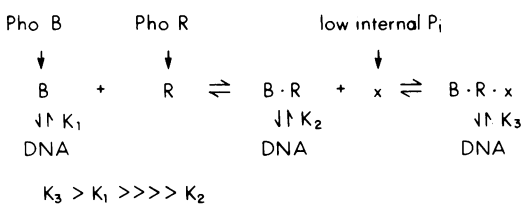


FIG. 7. *Model for regulation of alkaline phosphatase. K refers to an affinity constant between protein and promoter region of DNA.*

proteins and not bind x; these mutants could not be derepressed. Another kind of *phoR* mutant might respond differently to the x inducer. In this regard, it is of interest to note that Wilkins showed that mutational alterations in the *phoR* gene product could lead to induction of alkaline phosphatase by different metabolites, and he reported that one *phoR* mutant was inducible by starvation for purines to the full constitutive level in contrast to *phoR*$^+$ which was only partially inducible by purine starvation (23).

Certain features of this model are analogous to the molecular events involved in catabolite repression. Enzymes under control of catabolite repression require the presence of CRP (cyclic adenosine 5'-monophosphate [AMP]-binding) protein and a high level of cyclic AMP to be derepressed, regardless of the presence of specific inducers. Alkaline phosphatase formation is not affected by cyclic AMP per se but the "B-R" complex could be the equivalent of CRP and x inducer analogous to cyclic AMP. Finally, like the catabolite repression system, the alkaline phosphatase regulatory system is pleiotropic and regulatory genes affect the biosynthesis of at least three bacterial proteins that are localized to the bacterial cell periplasm.

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