JOURNAL OF BACTERIOLOGY, July 1976, p. 656-659

Vol. 127, No. 1
Printed in U.S.A.

NOTES

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

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Received for publication 17 February 1976

In phosphate-starved cells of *Escherichia coli*, the synthesis of alkaline phosphatase and some additional periplasmic proteins is derepressed. One of these proteins, which does not appear in a *phoS*$^-$ constitutive strain, has been identified as the periplasmic phosphate-binding protein.

Alkaline phosphatase (EC 3.1.3.1) of *Escherichia coli* is a periplasmic enzyme and its synthesis is strongly derepressed when the cells are starved for inorganic phosphate (P_i). The enzyme is a dimer (molecular weight, 86,000) composed of two identical monomers (15). It has been recently shown that starvation for P_i results in the derepression of three additional, and as yet unidentified, periplasmic proteins (13, 20). The known genes involved in the synthesis and control of alkaline phosphatase are *phoA*, *phoB*, *phoR*, *phoS*, *and phoT*. *phoA* is the structural gene coding for the enzyme subunit; *phoB* and *phoR* are regulatory genes and are closely linked to *phoA* at position 10 on the *E. coli* chromosome (4, 5, 6, 14, 19). *phoS* and *phoT* are linked to each other and are located remotely (position 74) from the *phoA*, *phoB*, *phoR* cluster (2, 6, 14). A mutation in *phoR*, *phoS*, or *phoT* results in constitutive synthesis of alkaline phosphatase. Recently evidence has been accumulated indicating that the two remote genes (*phoS* and *phoT*) are involved in P_i transport (11, 16, 17). It has also been shown that *phoS*$^-$ mutants lack a periplasmic P_i-binding protein (9) (also known as the R2a protein [8]), whereas *phoT*$^-$ mutants possess it. To test whether the P_i-binding protein corresponds with one of the unidentified periplasmic proteins co-regulated with alkaline phosphatase, we have isolated a series of strains that are constitutive for alkaline phosphatase synthesis. By conjugation matings between these strains and two Hfr strains, the position of each constitutive mutation was determined. The constitutive mutations showed strong linkage either to *proC* (position 10, *phoR*$^-$ mutation) or to *ilv* (position 74, *phoS*$^-$ or *phoT*$^-$ mutation) (data not shown). Three of the constitutive strains, as well as their parental "wild type" strain (CSH57A, see footnotes of Table 1), were grown in excess P_i and under P_i starvation. Enzyme activity in each of the

TABLE 1. *Alkaline phosphatase activity in constitutive strains*

Strain	*pho* genotype	Alkaline phosphatase activity (enzyme units)[a]	
		High P_i	Low P_i
CSH57A	Wild type[b]	0	1.8
CON10-1	*phoR*[c]	5.2	3.6
CON74-4	*phoS*[c]	2.9	3.1
CON74-5	*phoT*[c]	5.4	4.2

[a] One enzyme unit is defined as the change in absorbance at 410 nm that is equal to 1.0 per minute. Data are expressed as enzyme units divided by the culture absorbance (at 540 nm).

[b] Strain CSH57A has the following genotype: F$^-$ *ara leu lac proC purE gal trp his argG malA strA xyl mtl ilv metA* or *B*.

[c] Constitutive mutants were selected from strain CSH57A by treating the cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at pH 5.9 (1) and plating them for single colonies on tryptone agar plates. These plates contain sufficient P_i to repress alkaline phosphatase activity. The colonies were sprayed for alkaline phosphatase activity (3), and those showing enzyme activity were isolated and purified. For enzyme assays the cells were grown overnight at 37 C in medium A ("low P_i") (20) or in medium A supplemented with 1 mM KH_2PO_4 ("high P_i"). The cells were centrifuged and resuspended in 0.1 M tris(hydroxymethyl)aminomethane - hydrochloride buffer (pH 8.0) and assayed wth 1 mg of *p*-nitrophenyl phosphate per ml as previously described (20).

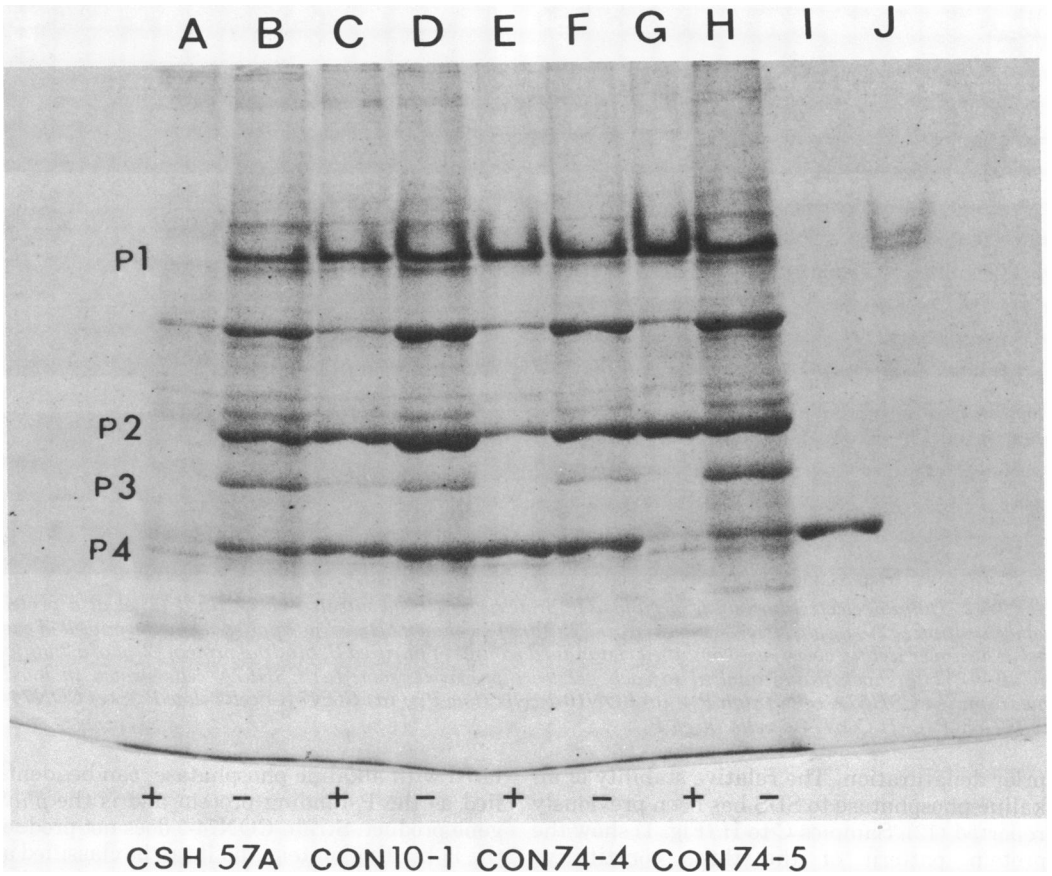

FIG. 1. *Pattern of periplasmic proteins obtained from strains grown in excess (+) and in low (−) concentrations of P_i. (A, B) Strain CSH57A; (C to H) three constitutive strains; (I) purified P_i-binding protein; (J) purified E. coli alkaline phosphatase. For the extraction of periplasmic proteins, overnight cultures were grown either in low P_i or in high P_i medium (see footnotes of Table 1), washed once with 0.1 M tris(hydroxymethyl)aminomethane-chloride buffer (pH 8.0) and suspended in one-fiftieth volume of the same buffer containing 20% sucrose. Lysozyme and ethylenediaminetetraacetic acid were added to a concentration of 0.1 mg/ml and 1 mM, respectively. After 60 min at room temperature the spheroplasts formed were centrifuged and the supernatant contained the periplasmic proteins. Protein concentrations were estimated by Hartree's modification of the Lowry method (10). Polyacrylamide slab gels containing 10% SDS were prepared as previously described (13). Approximately 30 μg of protein was applied in each slot. After electrophoresis, the gels were stained overnight with Coomassie blue and destained by the procedure of Fairbanks et al. (7). Approximately 2 μg of purified P_i-binding protein (9) and commercially purchased (Sigma) E. coli alkaline phosphatase were applied to slots I and J, respectively.*

cultures was measured (Table 1), and the periplasmic proteins were extracted by their release from cells converted to spheroplasts (13; see legend to Fig. 1). The proteins were applied untreated onto a sodium dodecyl sulfate (SDS)-polyacrylamide slab gel and separated by electrophoresis (Fig. 1). Samples A and B of the figure show the periplasmic proteins of the parental strain (CSH57A) grown in excess P_i and under P_i starvation, respectively. The bands labeled P1, P2, P3, and P4 are periplasmic proteins that appear in P_i-starved cells and that correspond to the proteins previously reported (13). From these gels, it is evident that some additional proteins appear as a result of P_i starvation. Band P1 is alkaline phosphatase in its dimeric form, because its position on the gel corresponds to a molecular weight of 86,000 (data not shown). Its mobility is the same as that of purified alkaline phosphatase (sample J in Fig. 1) and it does not appear in a *phoA*$^-$ deletion mutant (E15, data not shown). It is pertinent to note that in these SDS gels the samples were applied without

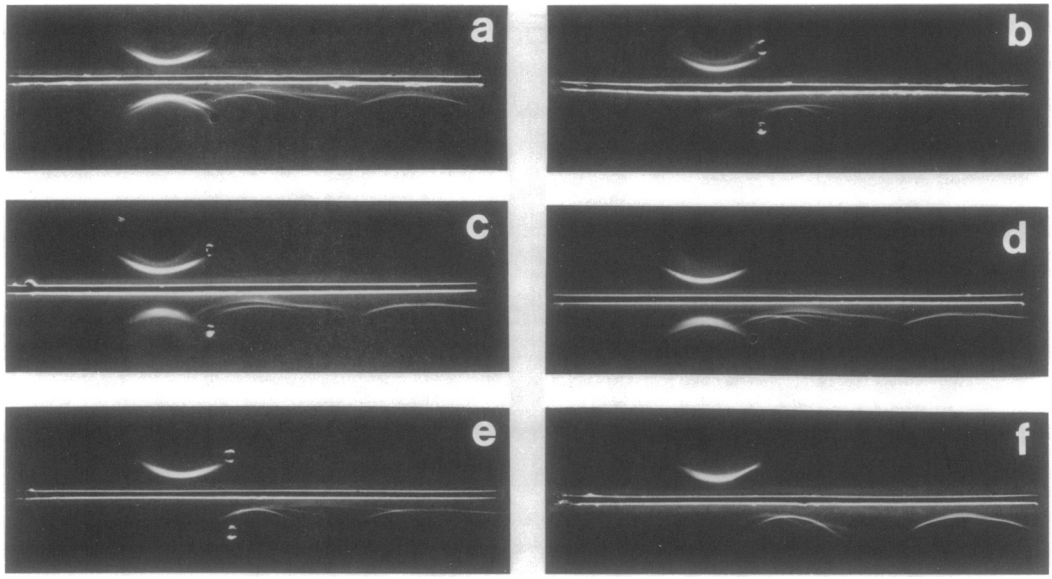

FIG. 2. *Immunoelectrophoresis of periplasmic proteins (9). Concentrated shock fluid (2 μl at a protein concentration of 20 mg/ml) from various strains cultured as shown below was applied to the lower well of each gel. The upper well in every case contained a standard sample of purified P_i-binding protein (2 μl of a 2 mg/ml solution). The shock fluids applied to each gel were prepared from: (a) CSH57A cells grown in low-P_i medium; (b) CSH57A cells (high P_i); (c) CON10-1 cells (low P_i); (d) CON74-4 cells (low P_i); (e) CON74-5 cells (low P_i); (f) CON74-5 cells (high P_i).*

prior denaturation. The relative stability of alkaline phosphatase to SDS has been previously reported (12). Samples C to H (Fig. 1) show the protein pattern of the three constitutive strains. CON10-1 is a *phoR*$^-$ strain and it produces alkaline phosphatase, P2 and P4 (and P3, partially) constitutively (samples C, D). Of the two strains that map at position 74, strain CON74-4 produces P2 and P4 constitutively (samples E, F), but in CON74-5 band P4 fails to appear in excess P_i, as well as in low P_i (samples G, H). The weak band that is seen at position P4 in strain CON74-5 (Fig. 1H) is a second protein (18 and see below). Sample I (Fig. 1) shows the purified P_i-binding protein; its position clearly coincides with that of P4. Therefore, band P4 is mainly composed of the P_i-binding (or R2a) protein. To strengthen this conclusion we have tested, in each of the strains, the cross-reactivity of the periplasmic proteins by immunodiffusion against antiserum prepared from the purified P_i-binding protein (Fig. 2). Only strain CON74-5, the one that is said not to possess P_i-binding protein, failed to show cross-reactivity (samples e and f in Fig. 2), although it showed precipitation arcs of other immunological systems that appear in the other strains.

We conclude that band P4, which is co-regulated with alkaline phosphatase, can be identified as the P_i-binding protein and is the *phoS* gene product. Strain CON74-5 does not produce the P_i-binding protein and it can be classified as a *phoS*$^-$ strain. Strain CON74-4, on the other hand, has the P4 band and, according to Echols et al. (6), it can be classified as a *phoT*$^-$ (or R2b) strain. The weak band that does appear at position P4 of strain CON74-5 (Fig. 1H) must be another protein for the following reason: in an earlier study (13), the periplasmic proteins of a wild-type strain were labeled with [^{35}S]methionine and showed a radioactive P4 band. Yet it is known that the P_i-binding protein does not contain any methionine residues (8, 9). Hence, position P4 must be occupied by a second protein, different from the P_i-binding protein. Both proteins are derepressed by starvation for P_i. Willsky and Malamy (18) have reached the same conclusions. They have further shown that, of the two proteins comprising band P4 (P4a and P4b), only one (P4b) can be labeled with [^{35}S]methionine, whereas the other (P4a, the P_i-binding protein) can be differentially released by treating the cells with polymyxin B sulfate.

We thank Gail Willsky and M. H. Malamy for conveying to us their results prior to publication. E. Y. was a recipient

of a grant from the United States-Israel Binational Science Foundation, Jerusalem, Israel.

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