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Isolation and Characterization of a New Type of *Escherichia coli* K12 *phoB* Mutants

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Summary. A new type of *Escherichia coli* K12 *phoB* mutant was isolated as 5-fluorouracil-plus-adenosine-resistant derivatives of a *upp phoS,T* strain. Such *phoB* mutants (called type III) differ from previously described *phoB* mutants (types I and II) in the synthesis pattern of phosphate-regulated periplasmic proteins P4a and 30.5 K, sensitivity to toxic compounds, and several other phenotypic characters. The analysis of isogenic strains carrying *phoB* mutations (type I, II or III) showed that; the *phoB* gene product exerted (i) a positive control over the synthesis of periplasmic proteins 30.5 K, 11.5 K, and 9 K, inner membrane proteins 32 K and 17.5 K, and outer membrane protein Tsx, (ii) and a direct or indirect negative control over the synthesis of a 20 K phosphate-inducible periplasmic protein.

Introduction

The *phoB* gene, located at 8.5 min on the *Escherichia coli* K12 linkage map, is a regulatory gene involved in the control of the phosphate regulon (Brickman and Beckwith 1975; Yagil et al. 1975). It is believed to encode for an activator protein that is necessary for the expression of structural genes coding for a group of phosphate-regulated proteins (Morris et al. 1974).

So far, two types of *phoB* mutation have been described. Type I-*phoB* mutants, which were previously called $phoR_c$, cannot derepress the synthesis of periplasmic proteins P1 (alkaline phosphatase monomer, molecular weight 45,000; 45 K) and P2 (*sn*-glycerol-3-phosphate binding protein; 40 K) (Argast and Boos 1980; Garen and Echols 1962; Willsky and Malamy 1976). Type II-*phoB* mutants do not derepress the synthesis of periplasmic proteins P1, P2 and P4a (phosphate-binding protein; 35 K) as well as protein PhoE, a major outer membrane protein (Morris et al. 1974; Tommassen and Lugtenberg 1980). Both types of *phoB* mutation are recessive to wild type and belong to the same complementation group (Kreuzer et al. 1975).

The syntheses of alkaline phosphatase and several other phosphate-regulated proteins are also indirectly controlled by two independent regulatory genes, *phoR* (Echols et al. 1961) and *phoM* (Wanner and Latterell 1980). The *phoR* gene product is a protein acting both as a negative and positive regulator, while the *phoM* gene product is a positive regulator (Tommassen et al. 1982; Wanner and Latterell 1980).

Control of the phosphate regulon is complex and not fully understood. The isolation of new allelic forms of its regulatory genes should help to explain its organization.

In this paper, we describe the properties of a third type of *phoB* mutants, which were selected as clones resistant to fluorouracil-plus-adenosine and deficient in alkaline phosphatase activity. A detailed comparative analysis of the three types of *phoB* mutation was carried out in isogenic strains. On the basis of our results, it is suggested that the *phoB* gene product could act, directly or indirectly, either as a positive or a negative regulatory element in the control of the various operons belonging to the phosphate regulon.

Materials and Methods

Bacterial Strains. All bacterial strains used in this work were *E. coli* derivatives. Some of their relevant properties are listed in Table 1.

Media. Cells were grown in rich L medium (Miller 1972), in minimal 63 medium (0.1 M phosphate) (Miller 1972) or Tris medium (T) (6.4 μM KH_2PO_4) (Worcel and Burgi 1974). Minimal media were adjusted to pH 7.0 and supplemented with 0.4% glucose (G), 1 μg per ml thiamine hydrochloride and appropriate amino acids. To derepress alkaline phosphatase synthesis, we used TG medium supplemented with 0.25% proteose peptone (TGLP medium, osmotic strength: 515 mos*M*). To repress alkaline phosphatase synthesis, we used TGLP medium supplemented with 10 mM KH_2PO_4 (TGHP medium, osmotic strength: 530 mos*M*). Osmotic strengths of media were determined with a cryometer (Prolabo) by measuring their freezing point.

Enzyme Assays. Quantitative assays for alkaline phosphatase and cyclic phosphodiesterase, using 3×3 mM *p*-nitrophenyl phosphate or 2 mM 3′-AMP as substrates, respectively, were performed by standard published procedures (Torriani 1967; Neu 1968). One unit of alkaline phosphatase activity is defined as the amount of enzyme that hydrolyzes 1 nmol substrate per min. For the 3′-nucleotidase activity of cyclic phosphodiesterase, one unit corresponds to the amount of enzyme that hydrolyzes 1 μmol substrate per h.

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Table 1. Bacterial strains

Strain	Genotype[a]	Source[b]/reference
Gal5	Hfr *thi metB1 lacI*	Lazzaroni and Portalier (1981)
49D	Hfr *thi metB1 lacI phoS, T deoC upp*	Heyde and Portalier (1982)
1455	Hfr *thi metB1 lacI phoS,T deoC upp phoB1455*	5-fluorouracil + adenosine-resistant derivative of strain 49D
1455R	Hfr *thi metB1 lacI phoS,T deoC upp*	Spontaneous 2 µg/ml chloramphenicol resistant revertant of strain 1455
LEA145	F⁻ *lacZ proC tsx trp rpsL xyl mtl*	Yagil et al. (1975)
LEA145.1	F⁻ *lacZ proC tsx trp rpsL xyl mtl upp*	Heyde and Portalier (1982)
LEP1	F⁻ *lacZ proC tsx purE trp rpsL xyl mtl phoB23*	Yagil et al. (1975)
LEP1.1	F⁻ *lacZ proC tsx purE trp rpsL xyl mtl upp phoB23*	5-Fluorouracil-resistant transductant of strain LEP1 with P1 grown on strain 49D
1455.1	F⁻ *lacZ tsx trp rpsL xyl mtl upp phoB1455*	[c]
163	F⁻ *lacZ tsx trp rpsL xyl mtl upp phoB63*	[c]
117	F⁻ *lacZ tsx trp rpsL xyl mtl upp phoR17*	[c]
C86	HfrC *relA1 tonA22 pit10 spoT1* T2 *phoS21*	CGSC 5009

[a] Genetic nomenclature is from Bachmann and Low (1980)

[b] CGSC, *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn (BJ Bachmann, Curator)

[c] Strains 1455.1, 163 and 117 were selected among Pro⁺ recombinant colonies showing an alkaline phosphatase negative or constitutive phenotype, after infection of strain LEA145.1 with P1 bacteriophages grown on mutants 1455 (*phoB1455*, this study), S3 (*phoB63*, CGSC5257) or C5 (*phoR17*, CGSC4943), respectively

Preparation of Periplasmic Fractions. Cells grown at 37° C in TGLP or TGHP medium were harvested in stationary phase of growth and osmotic shocks were performed as described by Heppel (1971). In such conditions, less than 1% of β-galactosidase activity and more than 80% of alkaline phosphatase activity were released.

Polyacrylamide Gel Electrophoresis and Fluorography. The electrophoresis gel system used for analyzing periplasmic fractions was adapted from Laemmli (1970). The sodium dodecyl sulfate (SDS)-polyacrylamide slab gel was a 7%–17% acrylamide gradient 30 cm long. Electrophoresis was carried out at 10° C for 17 h at 110 V. Preparation and electrophoresis of membrane fractions, gel fluorography and film scanning were performed as already described (Heyde and Portalier 1982).

Chemicals. 5-fluorouracil, nucleosides, nucleotides, alkaline phosphatase substrate, SDS, and bisacrylamide were purchased from Sigma Chemicals Co., St Louis, Mo., USA. Acrylamide was obtained from BDH Chemicals Ltd, Poole, England.

Genetic Techniques. Transductions with bacteriophage P1*kc* were carried out as previously described (Miller 1972). For complementation and dominance analyses, diploid strains carrying the *phoB1455* allele on an F′ episome, were constructed (Miller 1972), by mating Hfr 1455 with *recA* derivatives of strains LEP1 and LEA145 as recipients and selecting for Lac⁺ Pro⁺ Ade⁺ Str^r or Lac⁺ Pro⁺ Str^r sex-ductants, respectively. The genotypes of F′ factors (*lac*⁺ *proC*⁺ *purE*⁺) were tested by transfer into appropriate *recA* recipient strains. Complementation and dominance studies were performed by testing sex-ductants for alkaline phosphatase activity with a qualitative test (Bracha and Yagil 1969).

Results

Isolation of Pleiotropic Alkaline Phosphatase-Negative Mutants

Mutants resistant to fluorouracil-plus-adenosine (or 3′-AMP or 5′-AMP) and deficient for alkaline phosphatase activity, were isolated after nitroso-guanidine mutagenesis of strain 49D (Heyde and Portalier 1982). Among these mutants, we identified two independent classes of strain, which genetically differed: (i) strains altered in the *napA* locus, whose properties have been described elsewhere (Heyde and Portalier 1982), (ii) strains altered in the *phoB* gene, whose properties are described below. Their analyses suggested that they define a new type of *phoB* mutant, that we call type III. Of the mutants deficient for alkaline phosphatase and resistant to the fluorouracil-plus-nucleoside or nucleotide mixtures, 90% were type III-*phoB* mutants, which indicated that the method used for their isolation was very selective. We have characterized 15 independent type III-*phoB* mutants; as mutant 1455 was chosen as the representative strain, we called them 1455-like mutants.

1455-like Mutants Carried a phoB Mutation

(i) Like type I-*phoB63* and type II-*phoB23* mutants, 1455-like mutants were unable to derepress the synthesis of periplasmic proteins P1 and P2 (Fig. 1, lanes 3, 4 and 5, Table 2) and major outer membrane protein PhoE (Fig. 2, lane 4), after growth with limiting concentrations of inorganic phosphate. (ii) They carried mutations localized by three factor-crosses between *proC* and *tsx* genes, very close to the *phoB phoR* cluster, as no Pro⁺ AP⁺ transductants could be identified by P1 transduction using these mutants as donors and strain LEP1 (type II-*phoB23* mutant) as a recipient (Table 3). The mutation carried by strain 1455 was named *phoB1455*. (iii) The *phoB1455* allele was found to be recessive to the wild-type allele. Moreover, *phoB1455* and *phoB23* mutations did not complement each other for alkaline phosphatase activity.

To compare more precisely the properties of the different *phoB* mutants, we have constructed isogenic strains by introducing various *phoB* mutations into the genetic background of parental strain LEA145.1 (Table 1). We called them 163 (carrying the type I-*phoB63* mutation), LEP1.1 (type II-*phoB23* mutation) and 1455.1 (type III-*phoB1455* allelic form).

1455-like Mutants Differed from Type I-phoB Mutants

Unlike strain 163 (type I) (Fig. 1, lane 3), strains 1455.1 and LEP1.1 (type II) (Fig. 1, lanes 4 and 5), did not synthe-

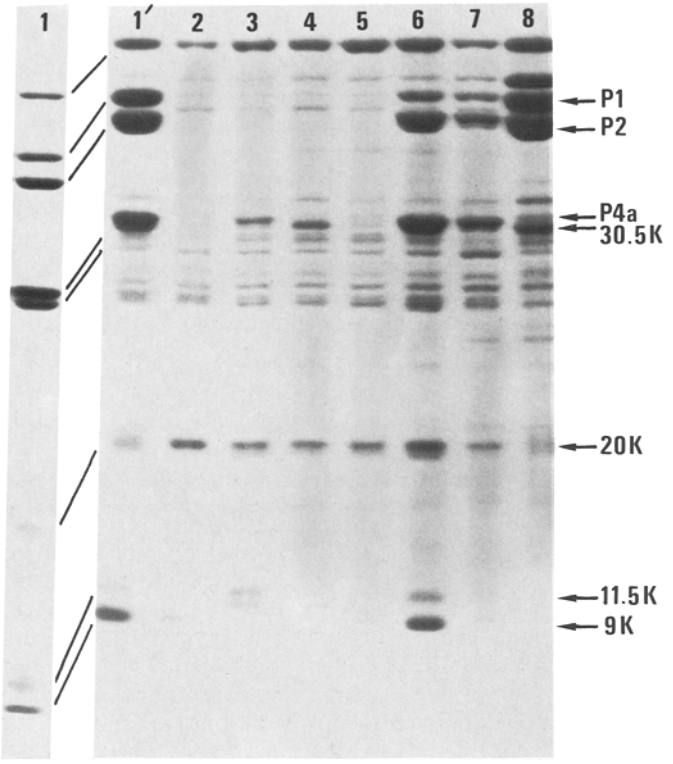

Fig. 1. SDS-polyacrylamide (7%–17% gradient) gel electrophoresis patterns of periplasmic proteins from parental, *phoB*, *phoR*, and *phoS* strains. Strains were grown in TGLP medium except where detailed. Samples of periplasmic proteins equivalent to 5×10^8 cells were subjected to electrophoresis. Standards: phosphorylase a (96 K); catalase (58 K); glutamic dehydrogenase (53 K); *E. coli* alkaline phosphatase (monomer 45 K); carbonic anhydrase (30 K); chymotrypsinogen (25 K) and lysozyme (14 K). Strains: LEA145.1 (lanes 1 and 1′); LEA145.1 (growth in TGHP medium) (lane 2); 163 (type I-*phoB63*) (lane 3); LEP1.1 (type II-*phoB23*) (lane 4); 1455.1 (type III-*phoB1455*) (lane 5); 117 (*phoR17*) (lane 6); 117 (*phoR17*, growth in TGHP medium) (lane 7) and C86 (*phoS21*) (lane 8)

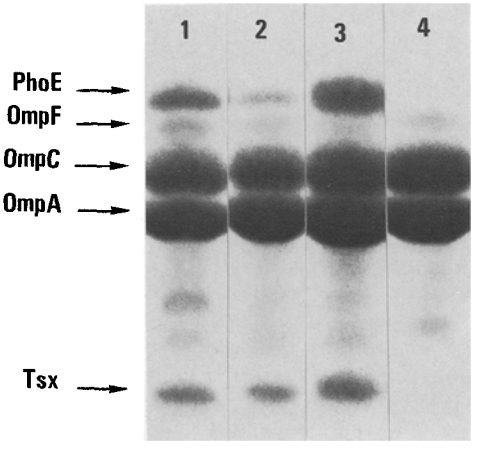

Fig. 2. SDS-polyacrylamide (10%) gel electrophoresis patterns of major outer membrane proteins of parental and *phoB1455* strains. Strains were grown at 37° C in TGLP medium, except when defined. Samples of total cell envelope proteins equivalent to 6×10^8 were subjected to electrophoresis. Only the relevant part of the gel was shown. After growth at 37° C, strains Gal5 and 49D were partially deficient for protein OmpF (data not shown). Strains: Gal5 (lane 1); Gal5 (growth in TGHP medium) (lane 2); 49D (lane 3) and 1455 (type III-*phoB1455*) (lane 4)

Table 2. Assays of some soluble enzyme activities in mutants 1455 and LEP1[a]

Strain	Sp. act. (units per mg bacterial dry weight)	
	Alkaline[b] phosphatase	Cyclic[c] phosphodiesterase
LEA145 (wild type)	155	8.6
LEP1 (type II-*phoB*)	1	7.8
Gal5 (wild type)	–	4.6
49D (wild type)	323	–
1455 (type III-*phoB*)	1	1.0

[a] Enzymatic activities were assayed in crude cellular extracts prepared as previously described (Heyde and Portalier 1982)
[b] Cells, grown in TGLP medium at 37° C, were harvested in late-exponential growth
[c] Cells were grown at 42° C in 63 medium. In such medium, alkaline phosphatase synthesis was repressed and the enzymatic substrate 3′-AMP was specifically hydrolyzed by cyclic phosphodiesterase

size periplasmic protein P4a after growth in low phosphate medium. Protein P4a was identified using a periplasmic extract from a *phoS* mutant (Fig. 1, lane 8) (Willsky and Malamy 1976).

1455-like Mutants Differed from Type II-phoB Mutants

Unlike strain LEP1.1 (type II) (Fig. 1, lane 4), strains 1455.1 and 163 (type I) (Fig. 1, lanes 3 and 5), never derepressed the synthesis of a periplasmic protein with an apparent molecular weight of 30.5 K, which migrated slightly faster than protein P4a. In the parental strain LEA145.1 and in a *phoR* mutant (Fig. 1, lanes 2 and 7), the synthesis of this protein was repressed by an excess of inorganic phosphate (10 mM KH_2PO_4). Protein 30.5 K could not be identical to protein P4b, since protein P4b synthesis was shown to be undetectable in a type II-*phoB* mutant and constitutive in a *phoR* mutant (Tommassen and Lugtenberg 1980). Moreover, protein P4b was not released by the osmotic shock procedure and was synthesized in very low amounts during growth of wild-type strains in the presence of limiting concentrations of inorganic phosphate (Willsky and Malamy 1976).

New Properties Common to all phoB Mutants

A comparative and detailed analysis of the three types of *phoB* mutant allowed us to identify new properties common to all *phoB* strains. (i) Two periplasmic proteins with apparent molecular weights of 11.5 K and 9 K were absent in strains 163, LEP1.1 and 1455.1 (Fig. 1, lanes 3, 4 and 5). The synthesis of both proteins was repressed after growth of wild-type and *phoR* strains in excess phosphate medium (Fig. 1, lanes 2 and 7). (ii) All *phoB* and *phoR* mutants tested displayed a partial constitutive synthesis of a peri-

Table 3. Genetic characterization of *phoB1455* mutation

Donor strain	Recipient strain	No. of Pro$^+$ recombinants analyzed[a]	Unselected characters[b]					
			AP$^-$	AP$^+$	T6^s		T6^r	
					AP$^-$	AP$^+$	AP$^-$	AP$^+$
1455 (type III-*phoB*)	LEA145.1 (wild-type)	127	76	51	59	2	17	49
1455	LEP1 (type II-*phoB*)	69	69	0	–	–	–	–

[a] Pro$^+$ recombinants were selected at 37° C on 63 medium plates supplemented with glucose and tryptophan, and purified twice by single colony isolation before further analysis

[b] T6^s, bacteriophage T6 sensitivity; T6^r, bacteriophage T6 resistance; AP$^+$, derepressible alkaline phosphatase activity; AP$^-$, no alkaline phosphatase activity. Phage T6 sensitivity of transductants was tested by cross-streaking on L-agar plates. Alkaline phosphatase activity was detected on colonies using a qualitative test previously described (Bracha and Yagil 1969)

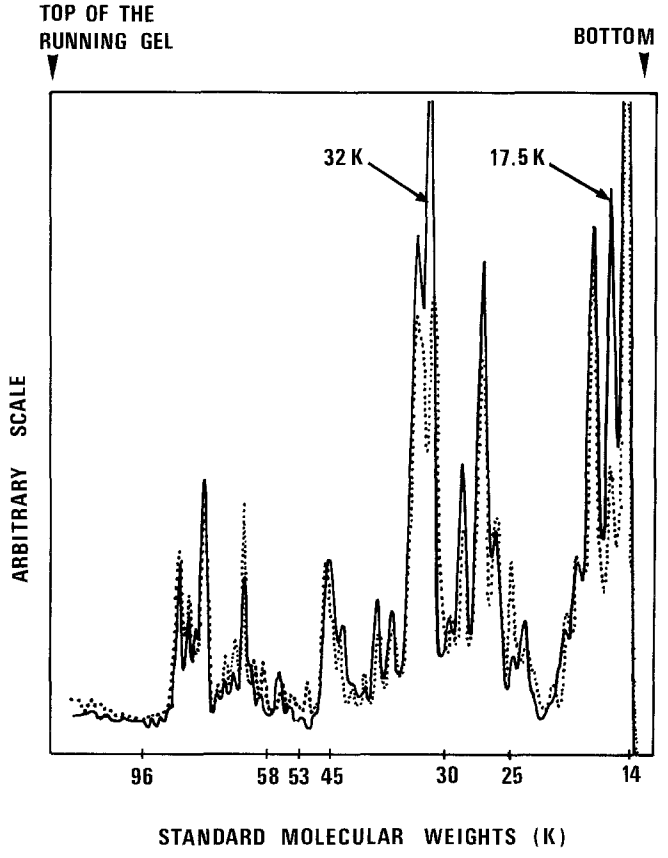

Fig. 3. SDS-polyacrylamide (10%) gel electrophoresis of inner membrane proteins from strains 49D (—) and 1455 (--). Proteins were labeled by growth with sulphate 35 in TGLP medium at 42° C (Heyde and Portalier 1982). The slab gel was 12 cm long. A sample of inner membrane proteins equivalent to 5×10^7 cells was subjected to electrophoresis. For autoradiography, gel exposure was 240 h. Molecular weights, as indicated, were determined using standards described in the legend to Fig. 1

plasmic protein with an apparent molecular weight of 20 K (Fig. 1, lanes 3, 4, 5 and 6). The synthesis of this protein was inducible in the parental strain LEA145.1 after growth in high phosphate medium (Fig. 1, lane 2).

Other Properties Associated with the Presence of a Type III-phoB Allele

phoB1455-like mutations conferred additional properties on their hosts. One outer and two inner membrane proteins of apparent molecular weights 27.5 K, 32 K, and 17.5 K, respectively, were synthesized in lower amounts by mutant 1455 than by strain 49D (Figs. 2 and 3). On the basis of its electrophoretic mobility, the 27.5 K major outer membrane protein was identified as protein Tsx. This was confirmed by a two-fold increased resistance of mutant 1455 towards bacteriophage T6. The increased resistance of 1455-like mutants to the fluorouracil-plus-adenosine mixture could be the consequence of a lowered expression of the *tsx* gene, as protein Tsx is known to be involved in nucleoside transport across outer membrane (Hantke 1976). The effect of the *phoB23* allele on the protein Tsx content could not be analyzed in strain LEP1.1, as it was not possible to transduce it to Tsx$^+$ phenotype.

We have shown that the synthesis of protein Tsx was not phosphate-regulated in wild-type strain Gal5 (Fig. 2, lanes 1 and 2) (Nesmeyanova et al. 1981).

Mutant 1455.1 was resistant to 60 μg 5-fluorouracil per ml and 10 μg 5-fluorouracil per ml plus 2 mM adenosine (or 3′-AMP or 5′-AMP), whereas the parental strain LEA145.1 and other *phoB* mutants (types I and II) were only resistant to 25 μg fluorouracil per ml and sensitive to fluorouracil plus adenosine. Similar patterns of resistance and sensitivity were observed when the *phoB1455* mutation was present in the genetic background of strain 49D.

Mutant 1455.1 was more sensitive than parental strain LEA145.1 to mitomycin C, chloramphenicol, EDTA, and rifampicin (data not shown). Strain LEP1.1 (type II) however, was more sensitive to rifampicin than strains LEA145.1 and 1455.1. Revertants of mutant 1455 were isolated as colonies resistant to chloramphenicol (2 μg/ml) with a frequency of 10^{-8}. Of them 94% displayed the parental phenotype which suggested that the *phoB1455* mutation responsible for a pleiotropic phenotype, was a single mutation.

The cyclic phosphodiesterase activity of mutant 1455 was 80% reduced, when compared with parental strain Gal5 (Table 2). It was not altered in strain LEP1 (type II), compared with parental strain LEA145. This property of

1455-like mutants, might contribute to their increased resistance to the fluorouracil-plus-AMP mixture.

When the *phoB1455* mutation was carried by an Hfr strain (49D), the conjugation efficiency of the donor was decreased, as qualitatively estimated by direct mating on plates.

Discussion

Inorganic phosphate starvation induces the synthesis of many exported proteins in *E. coli* (Morris et al. 1974; Tommassen and Lugtenberg 1980). The synthesis of a group of phosphate-regulated exported proteins is directly or indirectly regulated by genes controlling induction of alkaline phosphatase; they are three regulatory genes (*phoR, phoM,* and *phoB*) and several structural genes involved in phosphate transport (*phoS, phoT, pit,* and *pst*) (Tommassen et al. 1982; Wanner and Latterell 1980; Amemura et al. 1982). Several models have been proposed to explain the regulatory mechanisms involved in the expression of phosphate-regulated genes (Morris et al. 1974; Tommassen et al. 1982; Wanner and Latterell 1980). According to the more recent model (Tommassen et al. 1982), the *phoR* gene product would act on the *phoB* gene expression, either as a positive regulator under conditions of phosphate limitation or a negative regulator in excess phosphate. The *phoR* activator form can be substituted for by the *phoM* gene product (Tommassen et al. 1982; Wanner and Latterell 1980), while the *phoB* gene product is considered only as an activator for transcription of the *pho* regulon.

The results described in this paper, showed that the functions exerted by the *phoB* gene product might be more complex than previously assumed. First, we extended its positive control to three additional phosphate-regulated periplasmic proteins 30.5 K, 11.5 K, and 9 K. Second, we showed that the *phoB* gene product positively controls the synthesis of the outer membrane protein Tsx, whose synthesis is not phosphate-regulated. Thirdly, our results suggest that the *phoB* gene product might exert a direct or indirect negative control over the synthesis of a 20 K phosphate-inducible periplasmic protein. It should be noted that three periplasmic proteins called Pα, Pβ and Pγ were previously reported to be induced in wild-type *E. coli* strains, during growth in excess phosphate. But unlike protein 20 K, their production was shown to be independent of the known alkaline phosphatase control genes (Willsky and Malamy 1976).

The identification of new properties of the *phoB* gene, which we have described in this paper, was favored by the isolation of a new allelic form of the *phoB* gene and by comparing cell envelope contents of different *phoB* isogenic mutants with long gel gradients, allowing separation of low molecular weight proteins, which migrate at the front of standard gel systems. Moreover, we have shown that the synthesis of newly identified proteins (30.5 K, 11.5 K, 9 K, and 20 K) were really controlled by the phosphate level of the growth medium and not by the osmolarity of the medium, since the osmotic strengths of low and high phosphate media were equivalent.

Analogy between the roles of the *phoB* gene product and the CAP protein (adenosine 3′,5′-cyclic phosphate activator protein) has already been proposed by several authors (Morris et al. 1974; Wanner and Latterell 1980). Such a comparison is strengthened by our results and recent results of Mallick and Herrlich (1979), which have shown that the CAP-$_c$AMP complex might exert, in addition to its positive role, a negative role over the synthesis of several *E. coli* proteins. Our results strongly suggest a similarity between the molecular events involved in catabolic repression and phosphate regulation.

Additional experiments are in progress to determine more precisely the control exerted by the *phoB* gene product on synthesis of the exported proteins.

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