MUTANTS AFFECTED IN ALKALINE PHOSPHATASE EXPRESSION: EVIDENCE FOR MULTIPLE POSITIVE REGULATORS OF THE PHOSPHATE REGULON IN *ESCHERICHIA COLI*

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ABSTRACT

The expression of alkaline phosphatase (the product of the *phoA* gene) in *Escherichia coli* is believed to be subject to both positive control by the *phoB* gene product and negative control by the *phoR* gene product. We have isolated a large number of PhoA$^-$ mutants in the *phoR*$^-$ genetic background. Among mutants altered in the positive control of alkaline phosphatase, some were *phoB* mutants; others had a mutation in a new gene, designated *phoM*. We believe that the *phoM* gene codes for a positive regulator that acts together with the *phoB* gene product in *phoA* gene expressions.——The PhoM phenotype was found to be masked in *phoR*+ strains. This and other evidence support a positive regulatory role for the *phoR* gene product as well.——Our experiments demonstrate that *phoA* is under positive control by three different positive regulators: the products of the *phoB*, *phoM* and *phoR* genes. The *phoB* gene product is always needed together with either the *phoR* or *phoM* gene product. In addition, the *phoR* gene product acts as a negative regulator.——We describe a model for *phoA* gene expression consistent with this new evidence.

THE synthesis of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC3.1.3.1) in *E. coli* is induced several hundred-fold when the culture medium becomes depleted of inorganic monophosphate (HORIUCHI, HORIUCHI and MIZUNO 1959; TORRIANI 1960). The alkaline phosphatase structural gene, *phoA*, is located at 8.5 minutes on the *E. coli* chromosome (BACHMANN, LOW and TAYLOR 1976). Two classes of mutants, mapping at other loci, have been isolated that affect the induction of the *phoA* gene: (1) PhoA$^+$ constitutive mutants, including *phoR*, *phoS*, *phoT* and *pst* mutants, which synthesize alkaline phosphatase in the presence of inorganic phosphate (GAREN and OTSUJI 1964; WILLSKY, BENNETT and MALAMY 1973); and (2) PhoA$^-$ (uninducible) mutants, such as *phoB* mutants, which are blocked in the induction of the *phoA* gene product (GAREN and ECHOLS 1962a; BRACHA and YAGIL 1973; BRICKMAN and BECKWITH 1975). PhoA$^+$ constitutive mutants might synthesize an altered repressor of the *phoA* gene, or they could affect *phoA* gene expression indirectly, perhaps by affecting the transport of inorganic phosphate (WILLSKY, BENNETT and MALAMY 1973). PhoA$^-$ mutants might lack an activator molecule required for induction of the *phoA* gene.

A class of mutants affecting the induction of the *phoA* gene has also been isolated; this class contains mutations very closely linked to the *phoA* gene (SARTHY and BECKWITH, unpublished results.) One such mutation, designated *pho-1003*(*Bin*)*phoA*$^+$ (WANNER, SARTHY and BECKWITH 1979), permits a high level of alkaline phosphatase synthesis that is largely *phoB* independent and partially constitutive in a wild-type strain (WANNER, unpublished results.) The PhoA$^+$(Bin) mutant contains an up promoter-like mutation of the *phoA* gene. It could have a mutated *phoA* promoter region, a DNA insertion sequence encoding a new promoter or a genetic fusion to the promoter of a nearby gene. A ϕ80pl*phoA*$^+$ transducing phage carrying the *pho-1003*(Bin)*phoA*$^+$ mutation has been constructed (WANNER, SARTHY and BECKWITH 1979). This phage has been helpful in classifying new PhoA$^-$ mutants.

We previously isolated a large number of mutants blocked in the expression of PhoA that had mutations unlinked to the PhoA structural gene. To do this, we mutagenized an *E. coli* K12 strain that carried *phoA* and *phoR* mutations. Subsequently, we screened bacterial colonies derived from such mutagenized cultures for mutants unable to synthesize alkaline phosphatase following infection with the ϕ80pl*phoA*$^+$ transducing phage. In this manner, we found numerous PhoA$^-$ mutants that contain an extragenic mutation affecting *phoA* gene expression. Such mutants might affect the transcription, translation, secretion or processing of the *phoA* gene product.

Our parental strain can be transduced to PhoA$^+$-constitutive using the ϕ80pl*phoA*$^+$ or the ϕ80pl*pho-1003*(Bin)*phoA*$^+$ phage. By testing each mutant with these two ϕ80pl*phoA*$^+$ transducing phage, we distinguished two mutant types: Type I, *phoB*-like mutants that could be transduced to PhoA$^+$ constitutive only when infected with the ϕ80pl*pho-1003*(Bin)*phoA*$^+$ phage; and Type II mutants, such as PerA (WANNER, SARTHY and BECKWITH 1979), that could not be transduced to PhoA$^+$ with either phage. Each mutant type was shown to be sensitive to ϕ80 phage.

A genetic analysis of some of the Type I mutants has revealed a new gene, *phoM*, which affects *phoA* gene expression. In this paper, we describe genetic studies suggesting that the *phoM*$^+$ gene product has a positive role in PhoA expression. Furthermore, we present evidence that the *phoR*$^+$ gene product acts as both a positive and negative regulator of the *phoA* gene. The *phoB*$^+$ gene product is also required for *phoA* gene expression, in agreement with earlier work (BRICKMAN and BECKWITH 1975). Mutants blocked in the expression of PhoA are also blocked in the expression of other phosphate-regulated genes. We discuss a model for *phoA* gene expression consistent with this new evidence that the *phoA* gene is under positive control by multiple positive regulators.

MATERIALS AND METHODS

Media: Culture media used have been previously described (WANNER, SARTHY and BECKWITH 1979). Supplements to minimal media were added as needed, according to WANNER, KODAIRA and NEIDHARDT (1977). Phosphate indicator plates were based on MOPS medium and in addition contained 1.5% agar, 0.2% glucose and either no added inorganic phosphate, 10^{-4} M potas-

sium phosphate or 2×10^{-3} M potassium phosphate. 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine (XP, Bachem) was used at 2×10^{-3} % as a phosphatase indicator (BRICKMAN and BECKWITH 1975). 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG, BACHEM) was used at 2×10^{-3}% as an indicator for β-galactosidase activity (MILLER 1972).

Bacterial strains: The genotypes and origins of bacterial strains are listed in Table 1. All are *E. coli* K12 derivatives. All strains are F⁻, unless otherwise indicated. Strains R4, R16 and R21 represent red-colored mutants isolated as described below. Strain R21 was discovered to be lysogenic for the φ80pl*phoA*+ phage. Lysogenization probably occurred while screening for mutant colonies. A spontaneously cured derivative was isolated as a white colony on phosphatase indicator plates. This derivative was denoted R21W. Kanamycin-resistant transductants were selected on LB agar plates containing 20 to 50 μg per ml of kanamycin sulfate (Sigma), following 2 hr of growth in LB containing 10 mM citrate to allow phenotype expression.

Bacteriophage strains: The isolation of the plaque-forming φ80pl*phoA*+ phage and its φ80pl-*pho-1003*(Bin)*phoA*+ derivative has been previously described (WANNER, SARTHY and BECKWITH 1979). The λp*phoA-lacZ*$_{8-12}$ phage carrying the *phoA-lacZ*$_{8-12}$ operon fusion was isolated

TABLE 1

Bacterial strains

Strain	Characteristics*	Source
CA7087	HfrH *proC221 thi rel-1*	F. JACOB
C3	*HfrC phoR3 tonA22 rel-1*	A. TORRIANI
I07010	*aroB*	E. BRICKMAN
XPH4	*lacZ524 phoA20 phoR2 rpsL trp thi*	J. BECKWITH
XPh24	*lacZ524 trp rpsL* del(*brnQ phoA proC phoB phoR*)*24 thi*	J. BECKWITH
3–60	*araD163* del(*lac*)*169 rpsL rel-1 proC*::Tn*5 thi*	S. MICHAELIS
R4	*lacZ524 phoA20 phoB4 phoR2 rpsL thi*	This study
R16	*lacZ524 phoA20 phoR2 phoM16 rpsL thi*	This study
R21	*lacZ524 phoA20 phoR2 phoM21 rpsL thi* lysogenic for φ80pl*phoA*+	This study
R21w	*lacZ524 phoA20 phoR2 phoM21 rpsL thi*	See text
BW442	*lacZ524 phoA20 phoR2 rpsL thi aroB*	WANNER, SARTHY and BECKWITH
BW454	*phoR2 rpsL thi aroB*	Lac+ transductant of BW442 with P1 grown on I07010
BW456	*phoR2 rpsL thi perA8*	Aro+ transductant of BW454 with P1 grown on mutant 2 (WANNER, SARTHY and BECKWITH 1979)
BW569	*phoR2 rpsL thi*	Lac+ transductant of XPh4 with P1 grown on BW456
BW632.9, 0.12	*lac proC*::Tn*5 rpsL thi phoM21*	Kanamycin-resistant transductant of R21w with P1 grown on 3–60
BW759.2, 0.8	*lacZ524 proC*::Tn*5 rpsL thi phoM21* lysogenic for λp*phoA-lacZ*$_{8-12}$	BW632.9. lysogenized with λp*phoA-lacZ*$_{8-12}$

* Abbreviations used are those of BACHMANN, LOW and TAYLOR (1976), except for *porA*, which was denoted by WANNER, SARTHY and BECKWITH (1979). The notation "del" indicates a chromosomal deletion mutation.

by the method of CASADABAN (1976; A. SARTHY, Ph.D. Thesis, Harvard Medical School, 1979). The λcIΔ(*int*)*9h80* phage was obtained from J. BECKWITH. The φ80*psu3*+ phage was obtained from S. COOPER.

Genetics: Bacteriophage P1 transductions, mutagenesis and phage techniques were adapted from MILLER (1972). Strains lysogenic for the λp*phoA-lacZ*$_{8-12}$ were isolated by adsorbing the phage with the bacteria at a multiplicity of infection of 0.1 for 10 min. Lysogens were selected on agar medium using λcIΔ(*int*)*9h80* to kill nonlysogens.

Isolation of mutants: Strain XPh4 was mutagenized with UV to yield 0.1% survival. Approximately 0.01 to 0.1% of the survivors were Mal$^-$, as indicated by spreading portions of the mutagenized cultures onto maltose tetrazolium agar medium. Following mutagenesis, the cultures were divided and grown overnight in tryptone yeast extract medium. Portions of individual cultures were spread onto indicator medium that could simultaneously and independently differentiate PhoA and Lac phenotypes. Bacterial colonies were subsequently tested for their ability to synthesize alkaline phosphatase and β-galactosidase after infection with a φ80pl*phoA*+ and φ80p*lacZ*+ transducing phage as described previously (WANNER, SARTHY and BECKWITH 1979). Colonies that were Lac+ and PhoA$^-$ exhibited a red color. These mutants were saved and retested.

Plate test for PhoA phenotype: There exist 4 different possible PhoA phenotypes: (1) The wild-type phenotype is PhoA+ repressible. Such a strain has *phoA*+ *phoB*+ *phoR*+ alleles and synthesizes high levels of alkaline phosphatase during inorganic phosphate starvation (this can occur with isolated bacterial colonies on solid medium containing a low concentration of inorganic phosphate). (2) PhoR, PhoS, PhoT and Pst mutants that are *phoA*+ *phoB*+ synthesize alkaline phosphatase constitutively in the presence of inorganic phosphate. This phenotype is referred to as PhoA+ constitutive. (3) Mutants that are PhoA$^-$ because of a mutation within the alkaline phosphatase structural gene are denoted PhoA$^-$ (*phoA*$^-$). (4) Mutants that are PhoA$^-$ because of an extragenic mutation that reduces *phoA* gene expression, *e.g.*, *phoB* mutants, are designated PhoA$^-$(*phoA*+ uninducible).

We have devised a reliable method to distinguish the various *phoA* phenotypes on agar medium. This test combines the use of the compound XP, which is hydrolyzed by phosphatases to produce a blue dye, with the use of the substrate for alkaline phosphatase p-nitro-phenylphosphate (p-NPP), which is hydrolyzed to produce a yellow color. This test employs MOPS agar plates containing XP and a suitable carbon source such as glucose. Potassium phosphate is added either at 10^{-4} M for low phosphate medium or at 2×10^{-3} M for high phosphate medium.

To test for the PhoA phenotype, cells are streaked on the agar medium. The test is more reliable when the strain to be tested is picked from a rich medium plate, and it is streaked for isolation onto the phosphatase indicator medium. Patch tests are less reliable. After the isolated colonies have reached 1 to 2 mm in diameter, the blue color is scored and a solution of 0.4% p-NPP in 1 M Tris, pH 8, is dripped onto the bacterial streak. When positive, yellow color development occurs within 1 to 2 min. Otherwise, the colonies are still white after 1 hr. The appearance of different classes of mutants is shown in Table 2. Appropriate control strains are simultaneously tested on each culture plate. This test is sufficiently accurate to differentiate PhoA+ constitutive mutants that synthesize different constitutive levels of alkaline phosphatase.

RESULTS

Identification of alkaline phosphatase negative (PhoA$^-$) mutants: Mutants defective in the expression of PhoA have been isolated by a procedure that precludes the isolation of mutants with an altered *phoA* gene. Approximately 150,000 colonies derived from UV-mutagenized cultures of strain XPh4, which is *lacZ phoA* and *phoR*, were screened for PhoA$^-$ mutants on indicator medium, which could independently distinguish the PhoA and Lac phenotypes of individual colonies. These colonies were infected with a mixture of φ80p*phoA*+

TABLE 2

Simple test for PhoA phenotype

PhoA phenotype*	Appearance on medium†			
	With low phosphate	Plus p-NPP‡	With high phosphate	Plus p-NPP
PhoA+ repressible	blue	yellow	white	white
PhoA+ constitutive	blue	yellow	blue	yellow
PhoA- (*phoA*-)	white	white	white	white
PhoA- (*phoA*+)	blue	white	white	white

* The phenotypes are described in the text.

† The blue color results from the hydrolysis of XP by very low levels of phosphatase activity. The yellow color is produced by the rapid hydrolysis of p-NPP by high levels of phosphatase activity.

‡ The color shown represents the color of individual colonies before and after testing with p-NPP as described in the text.

and ϕ80p*lacZ*+ phage. Those colonies that simultaneously exhibited a PhoA- and a Lac+ phenotype after infection were saved. (Details of this screening procedure are given by WANNER, SARTHY and BECKWITH 1979).

These mutants might be altered in a positive control gene product required for PhoA expression, *e.g.*, *phoB* mutants, or in a gene product that is required at some post-transcription/translation initiation step in *phoA* gene expression, *e.g.*, *perA* mutants (WANNER, SARTHY and BECKWITH 1979). These two types of mutants, here designated as Type I and Type II mutants, can be readily distinguished by their effect on a promoter-like mutation of the *phoA* gene, *pho-1003*(Bin)*phoA*+, which renders expression of the *phoA* gene largely independent of the *phoB* allele (SARTHY and BECKWITH, unpublished results; WANNER, SARTHY and BECKWITH 1979). The defect in PhoA expression in Type I mutants is largely abolished by the introduction of the *pho-1003* (Bin)-*phoA*+ genetic region; on the contrary, the defect in PhoA expression in Type II mutants, including *perA* mutants, is not overcome by the introduction of the *pho-1003*(Bin)*phoA*+ genetic region.

To identify PhoA- mutants as Type I or Type II, each mutant was transduced separately with each of the ϕ80 transducing phages: (1) ϕ80pl*phoA*+, which carries the wild-type *phoA*+ genetic region, or (2) ϕ80pl*pho-1003*(Bin)*phoA*+, which carries an up promoter-like mutation of the *phoA* gene. Transductants were selected for growth on β-glycerol phosphate minimal medium. On this medium, only bacteria that can synthesize a high constitutive level of alkaline phosphatase are able to grow (TORRIANI and ROTHMAN 1961). The parental strain XPh4 cannot grow on β-glycerol phosphate minimal medium unless transduced with either the ϕ80pl*phoA*+ or the ϕ80pl*pho-1003*(Bin)*phoA*+ phage. Type I mutant derivatives can grow only if transduced with the ϕ80pl-*pho-1003*(Bin)*phoA*+ phage, and Type II mutant derivatives cannot grow when transduced with either phage. To rule out phage resistance, each mutant type was tested with a ϕ80p*lacZ*+ phage. The parental and both mutant types can grow on lactose minimal medium only if transduced with the ϕ80p*lacZ*+ phage.

Among some 50 mutants found, about 30 were identified as Type I mutants. Eleven of these mutants have been analyzed in this work. Each of the Type I mutants was subsequently lysogenized with the ϕ80pl*phoA*$^+$ transducing phage. The amount of alkaline phosphatase synthesized in *phoA*$^+$ derivatives of the Type I mutants is reduced more than 100-fold when compared to the *phoA*$^+$ derivatives of the parental strain.

Evidence for a new PhoA positive regulator gene, phoM: Type I mutants could have an altered *phoB* gene. We tested this possibility by genetic linkage experiments. The order of the relevant genes in this region of this chromosome is *phoA proC phoB phoR* in a clockwise orientation. The *phoA* gene is about 90% linked to *proC* by bacteriophage P1 transduction; the *phoB* gene is about 50% linked to *proC*. To test for the linkage of the mutations in the Type I mutants to *proC*, bacteriophage P1 was grown on several independent Type I mutants. These phage lysates were used to transduce strain CA7087 (*phoA*$^+$ *pro-C*$^-$ *phoB*$^+$ *phoR*$^+$) to proline independence. Forty ProC$^+$ transductants from each transduction were purified and then tested for their PhoA phenotype, using phosphatase indicator plates (see MATERIALS AND METHODS). Since the mutants were isolated in a *phoA*$^-$ *proC*$^+$ *phoB*$^+$ *phoR*$^-$ strain, most of the transductants became *phoA*$^-$. Among the ProC$^+$ *phoA*$^+$ transductants of CA7087, roughly half would be expected to receive the tightly linked *phoB* and *phoR* alleles from the mutant used as a donor; the other half would retain the *phoB*$^+$ *phoR*$^+$ alleles of strain CA 7087. If a particular mutant is *phoB*$^-$, the two types of ProC$^+$ *phoA*$^+$ transductants expected would be: (1) *phoB*$^+$ *phoR*$^+$, *i.e.*, PhoA$^+$ repressible; and (2) *phoB*$^-$ *phoR*$^-$, *i.e.*, PhoA$^-$ (*phoA*$^+$ uninducible.) On the other hand, if the donor strain is *phoB*$^+$, the two types of ProC$^+$ *phoA*$^+$ transductants expected would be: (1) *phoB*$^+$ *phoR*$^+$; and (2) *phoB*$^+$ *phoR*$^-$, i.e., *PhoA*$^+$ constitutive. Of eleven type I mutants tested, seven yielded both PhoA$^+$ repressible and PhoA$^-$ (*phoA*$^+$ uninducible) transductants. These were likely *phoB* mutants. Surprisingly, four mutants yielded both PhoA$^+$ repressible and *PhoA*$^+$ constitutive transductants. These four mutants appeared to be *phoB*$^+$ and likely contained a mutation in a gene unlinked to the *proC phoB* region that leads to a *phoB*-like PhoA$^-$ phenotype. We designated this new gene *phoM*.

Further tests of the phoB *allele in Type I mutants:* The *phoB* allele present in some of the Type I mutants was substantiated by using bacteriophage P1 grown on three mutants to transduce a derivative of the *phoA proC phoB phoR* deletion strain XPh24 (BRICKMAN and BECKWITH 1975) to proline independence. First, strain XPh24 was lysogenized with the ϕ80pl*phoA*$^+$ phage to introduce the *phoA*$^+$ gene. In such a lysogen, the *phoA*$^+$ gene is transposed near the *att*ϕ80 locus on the *E. coli* chromosome (BACHMAN, LOW and TAYLOR 1976). By using such a lysogen as a recipient in a bacteriophage P1 transduction of the *proC* region, all the ProC$^+$ transductants remain *phoA*$^+$, and the PhoA phenotype of the selected ProC$^+$ transductants can be readily tested. Because of the deletion of the *proC phoB* region in the recipient, no crossover events can occur between these two genes, and all the selected ProC$^+$ transductants would acquire the *phoB* allele from the donor strain.

Strain R4 was believed to be a *phoB* mutant; strains R16 and R21W were believed to be *phoM* mutants. Bacteriophage P1 grown on mutants R4, R16 and R21W were each used to infect strain XPh24 lysogenic for ϕ80pl*phoA*$^+$. Forty ProC$^+$ transductants were purified and tested for their PhoA phenotype. All the ProC$^+$ transductants derived from mutant R4 were PhoA$^-$ (*phoA*$^+$ uninducible). This confirmed the presence of a *phoB*$^-$ allele in this strain. On the contrary, all the ProC$^+$ transductants became PhoA$^+$ constitutive when either mutant R16 or R21W was used as a donor. This demonstrated that mutants R16 and R21W were *phoB*$^+$. Furthermore, this implies that the *phoM* gene is not located near this region of the chromosome (because 40 of 40 became PhoA$^+$ constitutive).

We tested the Type I mutants for the presence of an amber suppressible PhoA$^-$ phenotype. To do this, we made each of the mutants lysogenic for the ϕ80pl-*PhoA*$^+$ phage. We infected these lysogens with the amber suppressor carrying phage, ϕ80p*su*3$^+$ (RUSSELL *et al.* 1970) on phosphatase indicator medium. Mutant R21 became *phoA*$^+$ when tested. This demonstrated that mutant R21 contains an amber suppressible *phoM* allele. (*phoM21*). The finding of an amber suppressible *phoM* allele constitutes evidence for a positive role for the *phoM* gene product in *phoA* gene expression. (The genetic criteria needed to establish a positive or negative role for a gene product in regulation of gene expression have been reviewed by BECKWITH and Rossow 1974.) When the ϕ80p*su*3$^+$ lysogens of R21 were purified and assayed for their level of alkaline phosphatase activity, the amount was similar to the amount of activity present in a *phoA*$^+$ derivative of the parental strain.

Effect of the phoR *allele on the PhoM phenotype:* We have shown that the *phoM* mutation reduces expression of the *phoA* gene carried by a ϕ80 transducing phage. Conceivably, the *phoA* gene on the phage could be subject to other controlling elements because of the genetic structure of the transducing phage. To eliminate this possibility, we introduced the *phoA*$^+$ gene into strain R21W by bacteriophage P1 transduction. To do this, we used bacteriophage P1 grown on strain 3–60 (*proC*::Tn*5* *phoA*$^+$ *phoR*$^+$) to infect strain R21W. Kanamycin-resistant transductants were selected. The results of testing the PhoA phenotype of these transductants are shown in Table 3. Most acquired the *phoA*$^+$ gene from

TABLE 3

Bacteriophage P1 transduction of the phoR$^+$ *gene into the* phoM *mutant*

Transductants*	PhoA phenotype†
1	PhoA$^-$ (*phoA*$^-$)
20	PhoA$^-$ (*phoA*$^+$)
16	PhoA$^+$ repressible

* Bacteriophage P1 grown on strain 3–60 was used to transduce strain R21w to kanamycin resistance. Of 40 kanamycin-resistant transductants tested, 37 were proline auxotrophs. Each of these was tested on phosphatase indicator plates. The number of transductants with each phenotype is shown.

† The PhoA phenotype of each transductant was determined as described in MATERIALS AND METHODS.

the donor. Twenty of the *proC*::Tn*5* *phoA*$^+$ transductants were PhoA$^-$. This is expected if *phoM* reduces the expression of the *phoA* gene in its chromosomal site. Surprisingly, 16 of the 36 *proC*::Tn*5* *phoA*$^+$ transductants were clearly PhoA$^+$; these 16 were PhoA$^+$ repressible. This is the phenotype of wild-type, *i.e.*, *phoR*$^+$ bacteria, suggesting that the presence of a *phoR*$^+$ allele masks the effect of the unlinked *phoM* mutant allele. To test this possibility, two of the presumed *proC*::Tn*5* *phoA*$^+$ *phoR*$^+$ transductants of R21W were transduced to proline independence, using bacteriophage P1 grown on a donor strain that is *proC*$^+$ *phoR*$^+$ *phoM*$^+$. (The *proC* and *phoR* genes are approximately 50% linked by bacteriophage P1 transduction.) The results are shown in Table 4. The observation that roughly half of the transductants became PhoA$^-$ implies that the PhoM phenotype was masked by the phoR$^+$ allele. Analogous results were obtained when the PhoM mutant R16 was similarly analyzed (data not shown). Corroborative evidence for the R21W derivatives was obtained by lysogenizing the *proC*$^+$ *phoA*$^+$ PhoA$^-$ transductants shown in Table 4 with the ϕ80p*su3*$^+$ phage. Each presumed *proC*$^+$ *phoA*$^+$ *phoR*$^-$ *phoM21* transductant became PhoA$^+$ constitutive upon lysogenization, while no effect of the presence of the ϕ80p*su3*$^+$ phage was observed with the *proC*$^+$ *phoR*$^+$ *phoM21* transductants.

Evidence for a dual role for the phoR *gene product*: The isolation of a *phoM*$_{am}$ mutant argues in favor of a positive role for the *phoM* gene product in PhoA expression. Because the PhoM phenotype is not observed in a *phoR*$^+$ derivative, this implies that the *phoR*$^+$ gene product can also act as a positive regulator. Apparently, the positive roles of the *phoM* and *phoR* gene products can substitute for each other. The *phoR*$^+$ *phoM* strains are also repressible for PhoA expression, suggesting that the *phoR*$^+$ gene product has both a positive and a negative role in PhoA expression. On the other hand, the *phoM* gene product has only a positive role because a *phoR phoM*$^+$ strain is constitutive.

It might be possible to isolate *phoR* mutants that have abolished both the positive and negative function of the *phoR* gene product, or that have abolished only one of these functions. Most *phoR* mutants are known to synthesize a constitutive level of alkaline phosphatase equivalent only to 20 to 25% of the level

TABLE 4

Bacteriophage P1 transduction of the phoR2 *allele into the* phoM *mutant strain**

Recipient	PhoA phenotype†	
	PhoA$^+$ repressible transductants	PhoA$^-$ (*phoA*$^+$) transductants
BW632.9‡	9	7
BW632.12	7	5

* ProC$^+$ transductants were selected following infection with bacteriophage P1 grown on BW569.

† The phoA phenotype of the transductants was determined using phosphatase indicator plates as described in MATERIALS AND METHODS.

‡ Sixteen ProC$^+$ transductants of BW632.9 and 12 ProC$^+$ transductants of BW632.12 were tested. The numbers of transductants with each phenotype are shown.

of alkaline phosphatase synthesized during inorganic phosphate deprivation of a *phoR*$^+$ strain. (ECHOLS *et al.* 1961). This low constitutive level of alkaline phosphatase found in most *phoR* mutants (including amber suppressible *phoR* mutants) cannot be increased by phosphate starvation. Here, we have shown that the PhoA$^+$ constitutive expression found in such *phoR* mutants is dependent upon a *phoM*$^+$ allele. Such *phoR* mutations have likely abolished both the positive and negative function of the *phoR* gene product.

A few *phoR* mutants have been isolated that synthesize the higher level of alkaline phosphatase normally found during inorganic phosphate starvation (ECHOLS *et al.* 1961; GAREN 1963; MORRIS *et al.* 1974). This observation has led GAREN and ECHOLS (1962a) to suggest both a positive and negative role of the *phoR*$^+$ gene product in PhoA expression. The *phoR* mutant C3 (ECHOLS *et al.* 1961) synthesizes the higher level of alkaline phosphatase. The *phoR3* mutation in this strain has been shown to be recessive to *phoR*$^+$. The *phoR3* allele is also not complemented by the *phoR8* allele that results in synthesis of the lower constitutive level of alkaline phosphatase (KREUZER, PRATT and TORRIANI 1975). The *phoR3* allele, therefore, might code for a gene product that has lost the negative function but has retained the positive function of the *phoR* gene product. If so, then transfer of the *phoR3* allele into a *phoM* mutant should result in a PhoA$^+$ constitutive strain. To test this possibility, we used bacteriophage P1 grown on strain C3 to infect strain BW632.9 (*proC*::Tn*5* *phoR*$^+$ *phoM21*). ProC$^+$ transductants were selected on glucose minimal medium. Eight transductants were purified and tested for their PhoA phenotype on phosphatase indicator plates: two remained PhoA$^+$ repressible, the phenotype of the recipient strain BW632.9; six became PhoA$^+$ constitutive, the predicted phenotype of a *phoR3* *phoM*$^-$ strain if the *phoR3* gene product retains the positive function of the *phoR* gene product. The finding of PhoA$^+$ constitutive transductants of the *phoM* mutants using the *phoR*3 donor strain and finding PhoA$^+$ repressible transductants of the *phoM* mutants using the *phoR*$^+$ donor strain constitutes strong evidence that the *phoR* gene product acts as both a positive and negative regulator of the *phoA* gene.

Effect of phoM *and* phoR *alleles on transcription/translation initiation of the* phoA *gene:* Genetic fusions have been isolated that fuse the β-galactosidase structural gene to the *phoA* promoter-regulatory region (SARTHY *et al.* 1979). In strains carrying such fusions, the synthesis of β-galactosidase can be used as a measure of transcription/translation initiation of the *phoA* gene. When a strain that is *lacZ* is lysogenized with a bacteriophage lambda-transducing phage carrying the fusion *phoA-lacZ*$_{8-12}$, the synthesis of both alkaline phosphatase and β-galactosidase is induced during inorganic phosphate deprivation. When a *phoR* mutant allele is transferred into such a lysogen, both enzymes are expressed constitutively (SARTHY and BECKWITH, unpublished results; WANNER, unpublished results.) These results demonstrate that the negative function of the *phoR* gene product somehow blocks the transcription/translation initiation near the *phoA* promoter-regulatory region. When the effect of a Type I mutation on the synthesis of β-galactosidase in such a fusion-bearing strain is examined, the

synthesis of β-galactosidase becomes uninducible (WANNER, SARTHY and BECKWITH 1979). These results demonstrate that the *phoB*$^+$ gene product is required for transcription/translation initiation near the *phoA* promoter-regulatory region.

To determine whether the positive functions of the *phoM* and *phoR* gene products were required for transcription/translation initiation of the *phoA* gene, we lysogenized strain BW632.9 (*lacZ*$^-$ *phoA*$^+$ *proC*$^-$ *phoR*$^+$ *phoM21*) with the λp*phoA-lacZ*$_{8-12}$ phage. In this strain, the *phoA-lacZ* fusion-carrying phage (which is Δ*att*) can integrate by homology into either the *phoA*$^+$ or the mutant *lacZ524* gene. Following infection of BW632.9 with λp*phoA-lacZ*$_{8-12}$, lysogenic derivatives were selected using λ*CI*Δ*int9h80* phage to kill nonlysogens. Each of several independent lysogens exhibited β-galactosidase and alkaline phosphatase synthesis on low, but not high, phosphate medium. This demonstrates that the positive function of the *phoR*$^+$ gene product affects transcription/translation initiation of the *phoA* gene. To demonstrate a similar role for the *phoM*$^+$ gene product, two of these independent lysogens, BW759.2 and BW759.8, were transduced to proline independence, using a bacteriophage P1 lysate of the *phoR2* strain BW569. The ProC$^+$ transductants were tested for synthesis of β-galactosidase and alkaline phosphatase. The results are shown in Table 5. In all cases, when the ProC$^+$ transductants became PhoA$^-$ because of introduction of the *phoR2* allele, they became Lac$^-$ simultaneously. To confirm the presence of the *phoA-lacZ* fusion in these LacZ$^-$PhoA$^-$ transductants, eight ProC$^+$ transductants were infected with the φ80p*su3*$^+$ amber suppressor-carrying phage. Because the *phoM21* allele is amber suppressible, PhoA$^+$ constitutive transductants could be isolated in each case. Six of eight suppressed transductants became LacZ$^+$ constitutive, simultaneously. The other two lost the *phoA-lacZ* fusion-carrying phage, indicated by loss of bacteriophage lambda immunity. [Some loss of the fusion-carrying phage is expected because (1) these strains are partially diploid for both *phoA* and *lac* DNA, so that loss of the phage can occur by homologous

TABLE 5

Bacteriophage P1 transduction of the phoR2 *allele into the* phoM21 *strain lysogenized with* λpphoA-lacZ$_{8-12}$

Recipient	PhoA phenotype†	
	PhoA repressible transductants§	phoA+ PhoA$^-$ transductants‖
BW759.2‡	23	40
BW759.8	19	21

* ProC$^+$ transductants were selected following infection with bacteriophage P1 grown on BW569.

† The PhoA phenotype of the transductants was determined as in Table 3.

‡ Sixty-three ProC$^+$ transductants of BW759.2 and 40 ProC$^+$ transductants of BW759.8 were tested. The numbers of transductants with each phenotype are shown.

§ Twenty-two of the 23 PhoA$^+$ repressible transductants of BW759.2 were phosphate repressible for LacZ. One was LacZ$^-$. Sixteen of the 19 PhoA$^+$ repressible transductants of BW759.8 were phosphate repressible for LacZ; 3 were LacZ$^-$.

‖ All the *phoA*$^+$ *PhoA*$^-$ transductants were LacZ$^-$. See text for explanation.

recombination, and (2) the site of integration of the phage into either *phoA* or *lac* is linked to *proC* and some loss of the phage could occur by transduction of this region.] Table 5 also shows that most of the ProC$^+$ PhoA$^+$ repressible transductants retained the *phoA-lacZ* fusion, which was similarly repressible. These results imply that the positive role of both the *phoR* and *phoM* gene products affects transcription/translation initiation at the *phoA* promoter-regulatory region.

DISCUSSION

Mutants defective in the expression of the *phoA* gene have been isolated. Each of these mutants has a mutation unlinked to the *phoA* gene; these can be denoted as Type I or Type II mutants. Type I mutants appear to be regulatory mutants; the mutational defect in Type I mutants is overcome by the up promoter-like mutation of the *phoA* gene, *pho-1003*(Bin)*phoA*$^+$. Type II mutants do not appear to be affected in the regulation of the *phoA* gene; such mutants exhibit reduced expression of both wild-type *phoA*$^+$ and *pho-1003*(Bin)*phoA*$^+$ alleles. Type I mutants are also reduced in the expression of β-galactosidase controlled by a *phoA-lacZ* fusion, but Type II mutants are unaltered in the expression of a *phoA-lacZ* fusion (WANNER, SARTHY and BECKWITH 1979). These lines of evidence suggest that Type I mutants are blocked in transcription/translation initiation of the *phoA* gene and that Type II mutants are blocked in some subsequent step in *phoA* gene expression.

A genetic analysis of several independent Type I PhoA$^-$ mutants that were isolated in a *phoR*$^-$ strain revealed mutations in either of two genetic loci, *phoB* or *phoM*. The positive role for the *phoB*$^+$ gene product in PhoA expression is well documented (BRICKMAN and BECKWITH 1975). Several lines of evidence support a positive role for the *phoM*$^+$ gene product as well, in particular, the isolation of an amber suppressible *phoM*$^-$ mutant. The *phoM* mutations define a new genetic locus involved in the positive control of the *phoA* gene. Preliminary evidence suggests that the *phoM* gene maps near 0 minutes on the *E. coli* chromosome (WANNER, BERNSTEIN and LYSTER, unpublished results). We believe that *phoA* gene expression requires both a *phoB*$^+$ and *phoM*$^+$ gene product (at least in a *phoR*$^-$ genetic background).

The *phoM* mutants were isolated in a *phoR* strain. Introduction of the *phoR*$^+$ allele into the phoM mutants led to derivatives that were PhoA$^+$ repressible (see Table 4); these derivatives retained the original *phoM* allele. Thus, the PhoM$^-$ phenotype must be masked in the presence of the *phoR*$^+$ allele. Apparently, the *phoM*$^+$ and *phoR*$^+$ gene products can substitute for each other with respect to positive control of *phoA*. Transferring the *phoR*$^+$ allele into PhoM$^-$ mutants simultaneously restores both the inducibility and repressibility of PhoA, which implies that the *phoR* gene product has both a positive and a negative role in *phoA* gene expression. Corroborative evidence for a dual role of the PhoR product is provided by the demonstration that a particular *phoR* allele, *phoR3*, can abolish the negative function without affecting the positive function of the *phoR* gene product.

A model for PhoA expression has been previously proposed (MORRIS *et al.* 1974). This model attempted to explain the different types of *phoR* mutations by postulating an interaction between the *phoR* and *phoB* gene products. The discovery of the *phoM* gene has prompted us to propose a new model for *phoA* gene expression, which is depicted in Figure 1. According to this model, regulation of PhoA expression involves two events: repression and activation. Repression requires a *phoR* gene product (R) in a postulated repressor form, designated R^R. Repression can be abolished by either mutational alteration of the repressor (*phoR* mutations) or inorganic phosphate starvation. (Presumably, *phoS*, *phoT* and *pst* mutants elicit the same metabolic signal as actual inorganic phosphate starvation because *phoS,T* and *pst* are involved in inorganic phosphate transport.)

Activation of the *phoA* gene can occur upon relief of repression. This activation may be dependent on a postulated activator form of the *phoR* gene product (R^A), or it may be dependent on the *phoM* gene product (at least in the absence of a functional R product). The *phoR3* allele might alter R so that it always exists in the activator form. The *phoR* gene product, therefore, appears to act in a manner functionally analogous to that of the *araC* gene product in the regulation of the arabinose operon (WILCOX *et al.* 1974). The *phoR3* allele appears to act in *phoA* gene expression in a manner analogous to the $araC^c$ allele in expression of the arabinose operon.

In all cases activation of the *phoA* gene requires a $phoB^+$ gene product. (A $phoB^-$ $phoM^+$ $phoR^+$ strain is $PhoA^-$.) Possibly, B, the *phoB* gene product, acts in concert with either R^A or M to stimulate transcription initiation of the *phoA* gene. This could involve direct interaction between B and R^A or M or the interaction may be indirect. Perhaps B synthesizes a small-molecule effector that interacts with R and M in order to stimulate transcription. In this case, the mechanism for PhoA expression might be analogous to the adenosine 3′,5′-cyclic monophosphate (cAMP)-cAMP activator protein dependent system that is required for the expression of the *lac* operon and other catabolic genes in *E. coli* (SCHWARTZ and BECKWITH 1970). Alternatively, M or R^R might control the

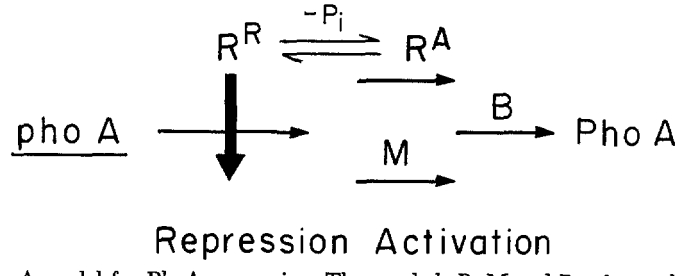

FIGURE 1.—A model for PhoA expression. The symbols R, M and B refer to the *phoR*, *phoM* and *phoB* gene products, respectively. R^R and R^A refer to the postulated repressor and activator forms of the *phoR* product as described in the text. The symbol $-P_i$ designates inorganic phosphate starvation. (The arrows have been drawn for the sake of simplicity. No temporal sequence is implied.)

synthesis of B, which in turn stimulates transcription of *phoA*. Additional studies are needed to test these and other possibilities.

The *phoM*$^+$ gene product appears to be responsible for the amount of alkaline phosphatase that is synthesized in most *phoR* mutants, but *phoM* does not appear to play any role in *phoA* expression in a *phoR*$^+$ strain during inorganic phosphate starvation. Other signals, such as purine starvation, can induce PhoA (WILKINS 1972). Possibly, these signals induce some change in R^R so that it can no longer repress PhoA, but such signals cannot elicit the metabolic signal necessary to convert R^R into the activator form, R^A. If so, PhoA expression would be PhoM dependent in response to these other signals. We are currently investigating this possibility.

Inorganic phosphate starvation also induces the synthesis of proteins other than alkaline phosphatase (MORRIS *et al.* 1974; WILLSKY and MALAMY 1976; YAGIL, SILBERSTEIN and GERDES 1976). The existence of several different unlinked phosphate-regulated genes implies the existence of a phosphate regulon similar to the glycerol regulon in *E. coli* (COZZARELLI, FREEDBERG and LIN 1968). The synthesis of some of these other phosphate-regulated proteins is *phoB* dependent. Perhaps particular phosphate-regulated proteins are *phoM* dependent, others *phoR* dependent and others, such as *phoA*, either *phoM* or *phoR* dependent. Studies of these other phosphate-regulated genes should be helpful in determining the role of multiple positive regulators in the expression of the phosphate regulon in *E. coli*.

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ADDENDUM

We have now demonstrated that the *phoM* gene is 90% linked to the *thr* locus in *E. coli* by bacteriophage P1 transduction. We have isolated deletion mutants that have simultaneously deleted *thr*, *phoM* and, in some cases, other genes in this region of the chromosome. These *phoM* deletion mutants have the same phenotype as the *phoM* point mutants reported here. The *phoM* deletion mutants are also suppressed by a *phoR*$^+$ allele (WANNER, BERNSTEIN and LYSTER, unpublished results).