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Regulation of the Phosphate Regulon of *Escherichia coli*: Analysis of Mutant *phoB* and *phoR* Genes Causing Different Phenotypes

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The *phoB* gene product of *Escherichia coli* is the transcriptional activator for the genes in the phosphate regulon as well as for *phoB* itself, all of which are induced by phosphate starvation. The *phoR* gene product modulates PhoB function in response to the phosphate concentrations in the medium. We quantitatively compared the levels of expression of the *phoA*, *phoB*, *phoE*, and *pstS* genes in several *phoB* mutants with different phenotypes by constructing operon fusions of these genes with the gene for chloramphenicol acetyltransferase. Although all the *phoB* mutants examined had little activator function for *phoA*, three among the four mutants showed various levels of the activator function for *phoB*, *pstS*, and *phoE*. To study the functional motifs of the PhoB and PhoR proteins, we cloned and sequenced the four classical *phoB* and six *phoR* mutant genes. All of the *phoB* mutations and one of the *phoR* mutations were missense mutations, and most of the altered amino acids were in the highly conserved amino acids among the regulatory proteins homologous to PhoB or PhoR protein, such as the OmpR, SfrA, and VirG proteins or the EnvZ, CpxA, and VirA proteins. The other five *phoR* mutations were nonsense mutations.

In *Escherichia coli*, genes in the phosphate (*pho*) regulon, including *phoA*, *pstS* (*phoS*), and *phoE*, which code for alkaline phosphatase (AP), phosphate-binding protein, and outer membrane porin e, respectively, are inducible by phosphate starvation and commonly regulated by multiple regulatory genes (for review, see references 26, 33, and 35). External phosphate levels are perceived by phosphate-binding protein, and the signal is transmitted by the functions of the products of the Pst-*phoU* operon (17) and the *phoR* gene to PhoB protein, which is the direct transcriptional activator for the genes in the *pho* regulon, including the *phoB-phoR* operon (17, 26). Although the molecular mechanisms of this process are largely unknown, the final outcome seems to be activation or deactivation of PhoB protein as the activator, depending on the scarcity or abundance of external phosphate. Makino et al. (11, 12) found a consensus sequence shared by the regulatory regions of *phoA*, *phoB*, *phoE*, and *pstS* which was named the *pho* box, and they showed that PhoB protein binds to the *pho* boxes of *pstS* and *phoB* and activates the transcription of *pstS* in vitro.

Several *phoB* mutants that do not produce AP have been isolated, and they have been used for studies of various aspects of the *pho* regulon. Among them, strains with a *phoB62* or *phoB63* mutation produced phosphate-binding protein, but the strain with the *phoB23* mutation did not (37). Guan et al. (8) reported that expression of *phoB* requires functional PhoB protein, but Shinagawa et al. (27) reported that expression of *phoB* was not much affected by *phoB19* or *phoB62* mutation. The leakiness of the *phoB* mutants might have been the cause of these discrepancies. To further characterize these "classical" *phoB* mutations, we assayed the levels of expression of *phoA*, *phoB*, *phoE*, and *pstS* in the four *phoB* mutants by constructing operon fusions of these genes with a reporter gene coding for chloramphenicol acetyltransferase (CAT).

The product of *phoR* can behave like either an activator or an inactivator of PhoB protein. This dual function was first suggested by the observation of two classes of mutants of *phoR*, one with a high constitutive level of AP (*phoR69*) and one with a low level (*phoR68*) (35). It was later found that another regulatory gene, *phoM*, which is not phosphate regulated, supplies the activator function lost in the *phoR68* mutant (36).

Here, we cloned and sequenced the mutant *phoB* and *phoR* genes to locate the mutation sites and to correlate the mutations with the phenotypes. We found that the missense mutations mostly lie within regions that are highly conserved among PhoB- or PhoR-like proteins, a family of two-component regulatory systems (18, 23, 38).

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used are listed in Tables 1 and 2, respectively. Nearly isogenic strains with a *phoB23* mutation or with a deletion in the *phoB-phoR* region were constructed as follows. pSN8011 was constructed by replacement of the 5-kilobase (kb) *Hpa*I fragment of pSN801 that contains the entire *phoB-phoR* operon (14; Fig. 1a) with the *Hin*cII fragment of pUC4K that contains the kanamycin resistance (Km^r) gene (34). pSN8011 was made linear by digestion with *Eco*RI and introduced into strain JC7623 (*recBC sbcB*; 20). Km^r transformants were selected, and recombinants with the deletion in the *phoB-phoR* region that was produced by homologous recombination between the chromosome and the cloned DNA on the plasmid (39) were confirmed by the AP^- phenotype. The deletion marker was transferred from one of the strains into the ANCK10 strain by transduction with P1 *vir* phage. The resultant *phoB-phoR* deletion derivative of ANCK10 was confirmed by an AP phenotype test and Southern blot analysis (29) and was named ANCH1. ANCL1, an ANCK10 derivative with a *phoB23* mutation, was similarly constructed by use of the cloned *phoB23* gene on pSNL801. The *Sal*I fragment containing the Km^r gene of pUC4K was inserted into the *Xho*I site of pSNL801, which is located upstream of the regulatory region of *phoB* (Fig.

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TABLE 1. Bacterial strains used in this study

Strains	Characteristics	Source/reference
ANCK10	F⁻ *leu lacY trp his argG strA ilv metA* (or *metB*) *thi*	(15)
ANCB19	As ANCK10 except for *phoB19*	(14)
ANCG206	As ANCK10 except for *phoB62*	(27)
ANCL1	As ANCK10 except for *phoB23* Km^r	This study
ANCS3	As ANCK10 except for *phoB63*	(15)
ANCH1	As ANCK10 except for Δ(*phoB-phoR*) Km^r	This study
ANCC22	As ANCK10 except for *phoR68 phoM453*	(15)
B19	Hfr(PO2A) *relA1 pit-10 spoT1 tonA22* T2^r *phoB19*	Laboratory stock
G206	Hfr(PO2A) *relA1 pit-10 spoT1 tonA22* T2^r *thi phoB62*	(10)
LEP-1	F⁻ *azi-6 tonA23? lacZ73 lacI22 pro tsx-67 purE42 supE44?* λ⁻ *trpE38 rpsL109 xyl-5 mtl-1 thi-1 phoB23*	(4)
S3	Hfr(PO2A) *ompF627 relA1 pit-10 spoT1 tonA22* T2^r *phoB63*	(10)
C2	Hfr(PO2A) *relA1 pit-10 spoT1 tonA22* T2^r *phoR68*	(6)
C3	Hfr(PO2A) *relA1 pit-10 spoT1 tonA22* T2^r *phoR69*	(6)
C5	Hfr(PO2A) *ompF627 relA1 pit-10 spoT1 tonA22* T2^r *phoR17*	(6)
C8	Hfr(PO2A) *relA1 pit-10 spoT1 tonA22* T2^r *phoR78*	(6)
C29	Hfr(PO2A) *ompF627 relA1 pit-10 spoT1 tonA22* T2^r *phoR19*	(7)
C36	Hfr(PO2A) *relA1 pit-10 spoT1 tonA22* T2^r *phoR20*	(7)

1a), giving pSNL8012. The linearized pSNL8012 was used to replace *phoB*$^+$ in strain JC7623 with the *phoB23* gene by homologous recombination. The *phoB23* gene was transduced from the JC7623 *phoB23* strain to ANCK10 by P1 *vir* phage, giving ANCL1.

Media and enzyme assays. LB medium and Tris-glucose media with high phosphate (TGHP) or low phosphate (TGLP) were as described previously (1). The AP phenotype test and the enzyme assay were performed as reported by Amemura et al. (1). CAT activity was assayed as described by Show (28).

DNA manipulation. Plasmid and phage M13 replicative form DNAs were prepared by the method of Birnboim and Doly (2). Restriction endonuclease digestion, agarose and polyacrylamide gel electrophoresis, in vitro ligation of DNA fragments with phage T4 DNA ligase, transformation, and transfection were done as described elsewhere (14).

Nucleotide sequencing. DNA was sequenced by the dideoxy chain-termination method (24, 25). Purified restriction fragments to be sequenced were cloned into the single-stranded DNA cloning vector M13mp18 or M13mp19 (40). JM103 (16) was used as the host strain.

Enzymes and radioisotopes. The restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, and an M13 sequencing kit including DNA polymerase (Klenow fragment) were obtained from Takara Shuzo (Kyoto, Japan). All enzymes were used as directed by the supplier. [α-^{32}P]dCTP was purchased from Amersham Japan (Tokyo).

RESULTS AND DISCUSSION

Expression levels of *pho* genes in various *phoB* mutants. The *phoB* mutants were all isolated as mutants that did not synthesize AP, and the expression of the *pho* genes is considered to require the function of the *phoB* gene, but some *phoB* mutants allow the expression of *pstS* (8, 37) or *phoB* (27). To study the effects of these *phoB* mutations on the expression of several *pho* genes, we constructed operon fusions between the promoter regions of the *phoA*, *phoB*, *phoE*, and *pstS* genes and a promoterless reporter gene (*cat*) coding for CAT that was carried on pKK232-8 (5). The promoter regions of these genes, which included the *pho* boxes, the −10 regions, and the transcription start sites (12, 32), were prepared from plasmids carrying these genes. The 64-base-pair *Pvu*II-*Rsa*I fragment carrying the *phoA* promoter region was obtained from pSN301 (19). The 205-base-pair *Acc*II fragment carrying the *phoB* promoter region was excised from pSN802 (13), and the cohesive ends were converted to blunt ends by T4 DNA polymerase. The 135-base-pair *Sau*3A1-*Hin*fI fragment carrying the *phoE* promoter region (21) was prepared from pPE600 (unpublished data), and its cohesive ends were converted to blunt

TABLE 2. Plasmids used in this study

Plasmids	Characteristics	Source/reference
pBR322	Amp^r Tc^r; vector for cloning	(3)
pKA064	Amp^r; pKK232-8 derivative carrying a *phoA* promoter region	This study
pKB205	Amp^r; pKK232-8 derivative carrying a *phoB* promoter region	This study
pKE135	Amp^r; pKK232-8 derivative carrying a *phoE* promoter region	This study
pKK232-8	Amp^r; vector for assay of promoter activity	(5)
pOS1	Amp^r; pKK232-8 derivative carrying a *pstS* promoter region	(9)
pSN801	Amp^r Tc^r; pBR322 derivative carrying a *phoB*$^+$-*phoR*$^+$ gene	(14)
pSN8011	As pSN801 except for having the Km^r gene instead of the *Hpa*I fragment (*phoB*$^+$-*phoR*$^+$)	This study
pSN802	Tc^r; pBR322 derivative carrying a *phoB*$^+$-*phoR*$^+$ region	(14)
pSNL801	As pSN801 except for carrying *phoB23*	This study
pSNL8011	As pSNL801 except for insertion of the Km^r gene into the upstream region of the *phoB* gene	This study
pUC201	Amp^r; pUC9 derivative carrying the *phoR*$^+$ gene	This study
pUC4K	Amp^r; plasmid for Km^r gene cartridge	(34)
pUC9	Amp^r; vector for cloning	(34)

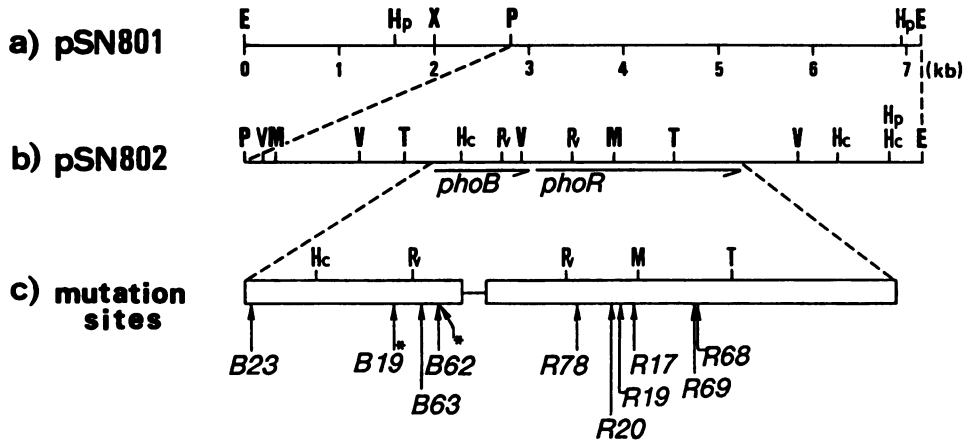

FIG. 1. Restriction map of (a) the 7.2-kb *Eco*RI fragment in pSN801 and (b) the 4.2-kb *Pst*I-*Eco*RI fragment in pSN802 carrying the *phoB-phoR* operon. *phoB* and *phoR* regions are indicated by the arrow. (c) Mutation sites and the allele number of each mutant gene identified by DNA sequencing are shown under the *phoB* and *phoR* genes (open boxes). Mutation sites of the *phoB19* allele, which has two mutations, are indicated by asterisks. One of them is at the same position as the one in *phoB62*. Restriction sites: *Eco*RI (E), *Eco*RV (V), *Hin*cII (Hc), *Hpa*I (H), *Mlu*I (M), *Pst*I (P), *Pvu*II, (Pv), *Tth*111I (T), and *Xho*I (X).

ends. Each of these DNA fragments was inserted into the *Hin*cII site of M13mp18. The nucleotide sequences and orientations of the inserts in the recombinant phages were analyzed by DNA sequencing. The *Bam*HI-*Hin*dIII fragment containing the promoter region of each gene in the desired orientation was recloned from the recombinant phages into the *Bam*HI-*Hin*dIII sites of pKK232-8 to give the operon fusion (Fig. 2). Plasmid pOS1, carrying a *pstS-cat* operon fusion, was similarly constructed previously (9).

The expression levels of *phoA*, *phoB*, *phoE*, and *pstS* were measured by assays of the levels of CAT activity in various *phoB* mutant strains carrying the plasmids with the operon fusions grown either in excess- or low-phosphate medium (Table 3). In all cases, expression of the *pho* genes in the wild-type strain was induced by phosphate limitation, but the induction ratios ranged from 10-fold for *phoB* to 200- to 300-fold for *pstS* and *phoA*. None of the *phoB* mutants examined here promoted the transcription of *phoA*. This was not unexpected, because all the *phoB* mutants had been isolated as ones that did not produce AP.

The *phoB23* mutation appeared to be a null mutation, because levels of expression of all of the *pho* genes in the ANCL1 strain examined here were similar to those in the ANCH1 [Δ(*phoB-phoR*)] strain. The expression of *phoA* and *pstS* was more strictly regulated by PhoB protein than that of *phoB* and *phoE*; low levels of residual expression of the latter genes were observed in the *phoB-phoR* deletion mutant. *pstS* was partially expressed in strains with a *phoB62*, *phoB63*, or *phoB19* mutation, and it was expressed very little in strains with a *phoB23* or *phoB-phoR* deletion. These results agree with those obtained by immunological methods with antisera against AP and phosphate-binding protein (37). Levels of *phoB* expression of the $phoB^+$ strain were about 20 and 30% in the *phoB19* and *phoB62* strains, respectively, and the expression of *phoB* in the *phoB23* strain was about 1%. These results confirm the finding by Guan et al. (8) that *phoB* transcription is positively regulated by PhoB protein.

It should be noted that *phoE* was expressed in the *phoB62* strain at the same level as in the $phoB^+$ strain, but *phoA* was not expressed in the mutant at all. In the *phoB-phoR* deletion mutant, *phoE* was expressed at a slightly higher level than the other three *phoB* mutants (*phoB19*, *phoB63*, and *phoB23*), and this residual expression was not regulated by the phosphate level. PhoB protein may negatively regulate the transcription initiated from a promoter other than the one identified by Tommassen et al. (32).

Cloning and sequencing of the mutant *phoB* and *phoR* genes. To analyze the alterations in the mutant genes by DNA sequencing, we cloned each of them into a plasmid vector. The 7.2-kb *Eco*RI fragment contains both *phoB* and *phoR* genes (14, 31), so we constructed a DNA library by digestion of the chromosomal DNA of each mutant with *Eco*RI and ligation of the digested DNA fragments with *Eco*RI-digested pBR322. The mutant *phoB* genes were cloned by selection of the $phoR^+$ gene, and the mutant *phoR* genes were cloned by selection of the $phoB^+$ gene. For cloning of the mutant *phoB* genes, a mixture of recombinant plasmids was transformed into strain ANCC22 (*phoR phoM*), and transformant colonies producing AP ($phoR^+$) were selected. For cloning of the mutant *phoR* gene, the

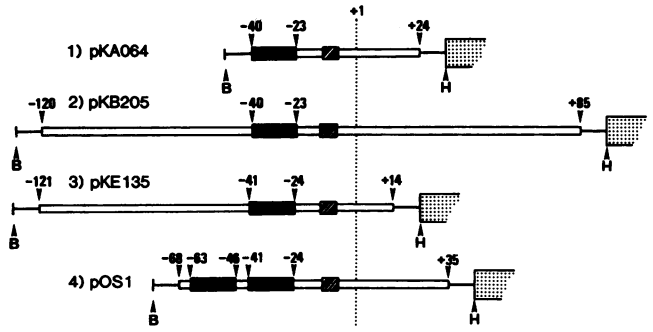

FIG. 2. Constructions of hybrid plasmids for analysis of promoter activity of the *pho* genes. (1) pKA064, (2) pKB205, (3) pKE135, and (4) pOS1 are pKK232-8 derivatives carrying the promoter regions of *phoA*, *phoB*, *phoE*, and *pstS*, respectively. The details of construction of each plasmid are described in the text. The numbers refer to the distances (in base pairs) relative to the position of the transcription starting point of each *pho* gene as described previously (12, 32). The transcriptional start points are indicated by the dotted line and +1. Open boxes, closed boxes, hatched boxes, and dotted boxes indicate the chromosomal segments, the positions of the *pho* boxes, the −10 regions, and the *cat* genes derived from pKK232-8, respectively. Solid lines indicate segments derived from pUC9. Symbols: B, *Bam*HI, H, *Hin*dIII.

TABLE 3. Expression levels of the *pho* genes in the *phoB* mutants[a]

Host strains	Vector (pKK232-8)		*phoAp* (pKA064)		*phoBp* (pKB205)		*phoEp* (pKE135)		*pstSp* (pOS1)	
	LP	HP	LP	HP	LP	HP	LP	HP	LP	HP
phoB$^+$ [ANCK10]	<0.2	<0.2	380	1.2	464	6.4	157	14	2138	12
phoB62 [ANCG206]	<0.2	<0.2	0.5	0.4	134	23	157	32	314	5.6
phoB19 [ANCB19]	<0.2	<0.2	1.2	0.1	108	43	17	6.7	90	18
phoB63 [ANCS3]	<0.2	<0.2	3.3	0.4	29	2.2	6.3	6.4	55	2.6
phoB23 [ANCL1]	<0.2	<0.2	0.5	0.5	4.1	2.2	7.0	6.1	1.0	0.6
Δ(*phoB-phoR*) [ANCH1]	<0.2	<0.2	0.5	1.0	6.4	2.8	20	23	0.8	0.7

[a] The levels of gene expression were monitored by measurement of CAT activity as described in Materials and Methods. CAT activity is expressed as nanomoles of 5-thio-2-benzoate liberated per minute per OD_{450} unit of the cell culture. Host strains with the indicated *phoB* genes are shown in brackets. Plasmids containing the *pho-cat* chimeric genes are indicated in parentheses. LP, TGLP liquid medium; HP, TGHP liquid medium.

plasmids were transformed into strain ANCB19 (*phoB*$^-$) and transformant colonies producing AP (*phoB*$^+$) were selected. The physical map of the recombinant plasmids carrying the mutant *phoB* or *phoR* gene was identical to that of pSN801 which contained the *phoB-phoR* region of the wild-type strain (14; Fig. 1). The 1.4-kb *Pst*I-*Hin*cII, 0.3-kb *Hin*cII-*Pvu*II, and 0.4-kb *Pvu*II-*Pvu*II fragments covering the *phoB* region (Fig. 1b) of the mutant were recloned into M13mp19 phages to be sequenced. The nucleotide sequences of the entire region of the mutant *phoB* genes, from the nucleotide 10 base pairs upstream of the *pho* box (12) to the beginning of the *phoR* coding region (13), were analyzed. All of the mutations were base substitutions; none were nonsense mutations (Table 4).

For the analysis of the mutant *phoR* genes, the 1.8-kb *Eco*RV fragment that covered the entire *phoR* gene (Fig. 1b) was isolated from each of the plasmids that contained the *phoB-phoR* region of the *phoR* mutants and pSN802 (*phoB*$^+$ *phoR*$^+$) and was recloned into the *Sma*I site of pUC9. The recombinant plasmid carrying the *phoR*$^+$ gene was designated pUC201. The DNA segments flanked with the *Eco*RI-*Pvu*II, *Pvu*II-*Mlu*I, or *Mlu*I-*Tth*111I sites of each plasmid (Fig. 1c) that carried the mutant *phoR* gene were replaced with the corresponding fragments of pUC201. The location of the mutations on these segments was identified by introduction of the recombinant plasmids into strain ANCC22 (*phoR phoM*) and testing the PhoR$^+$ phenotype of the transformants. We sequenced the DNA segments that carried mutations. All of the *phoR* mutations except the *phoR69* mutation were nonsense mutations. The results of sequencing are summarized in Table 4. The mutation sites found here were compared with those mapped by genetic crosses by Kreuzer et al. (10). The relative positions of *phoB62* and *phoB63*, *phoR20*, and *phoR78*, or *phoR68* and *phoR69* as assigned by Kreuzer et al. were found to be the opposite in our experiment.

TABLE 4. Base alterations and amino acid changes in the *phoB* and *phoR* mutants[a]

Allele no.	Mutation site	Amino acid change	Strain
phoB19	ACA → AAA	Thr-158 → Lys	B19
	CGC → TGC	Arg-201 → Cys	
phoB23	GAA → AAA	Glu-9 → Lys	LEP-1
phoB62	CGC → CAC	Arg-201 → His	G206
phoB63	GGA → AGA	Gly-185 → Arg	S3
phoR17	TATCTGA → TΔTCTGA	Tyr-155 → opal	C5
phoR19	CAG → TAG	Glu-141 → amber	C29
phoR20	TGG → TAG	Trp-135 → amber	C36
phoR68	TAC → TAA	Tyr-225 → ochre	C2
phoR69	ACC → AAC	Thr-220 → Asn	C3
phoR78	AAA → TAA	Lys-98 → ochre	C8

[a] The sequences of the *phoB*$^+$ and *phoR*$^+$ genes were determined previously (12, 13).

Missense mutations in *phoB* and *phoR* lie within domains highly conserved among the two-component regulatory proteins. The PhoB protein is homologous to a family of bacterial regulatory proteins that act as a part of the sensory and regulatory mechanisms responsible for phenotypic adaptation in response to environmental changes (18, 23, 38). In addition, each of the systems has a protein that functions as a sensor or transmitter of environmental signals and shares sequence homology with PhoR protein (22, 30). Since functionally important sequences of proteins are likely to be preserved through evolution, we expected that the mutations that affected the functions of PhoB or PhoR protein might alter the amino acids in the conserved sequences among these homologous proteins. In an examination of this possibility, the missense mutation sites of *phoB* and *phoR* identified here were found on the aligned homologous sequences shown in Fig. 3.

The *phoB23* mutation, which completely inactivated the PhoB function (Table 3), had a change in the ninth amino acid, where the negatively charged amino acids Glu or Asp are conserved, to an oppositely charged amino acid, Lys, suggesting that a negatively charged amino acid in this position may be essential for the function. Since the amino-terminal half of PhoB was suggested to be involved in the regulatory function of the carboxy-terminal activator function (26), the highly conserved region in the vicinity of the mutation site may be related to the interaction with PhoR for activation of PhoB protein.

phoB62 and *phoB19* mutations altered the same amino acid 201, where positively charged amino acids, Arg or His, are conserved. *phoB62*, which is the most leaky mutation analyzed here (Table 3), had a change from Arg to a similar amino acid, His; *phoB19*, which is less leaky, changed the Arg to an amino acid of a different group, Cys. Therefore, the more profound alteration in the chemical nature of the replaced amino acid in *phoB19* might be the cause of the functional defect. The carboxy-terminal half of PhoB was suggested to be involved in transcriptional activation and DNA-binding activity (26). The highly conserved region including this site might constitute one of the motifs for this activity.

The mutation site of *phoB63* changed Gly-185 to a positively charged amino acid, Arg. This amino acid is conserved only in three out of the five homologous proteins in the much less conserved region, yet still inactivated the function

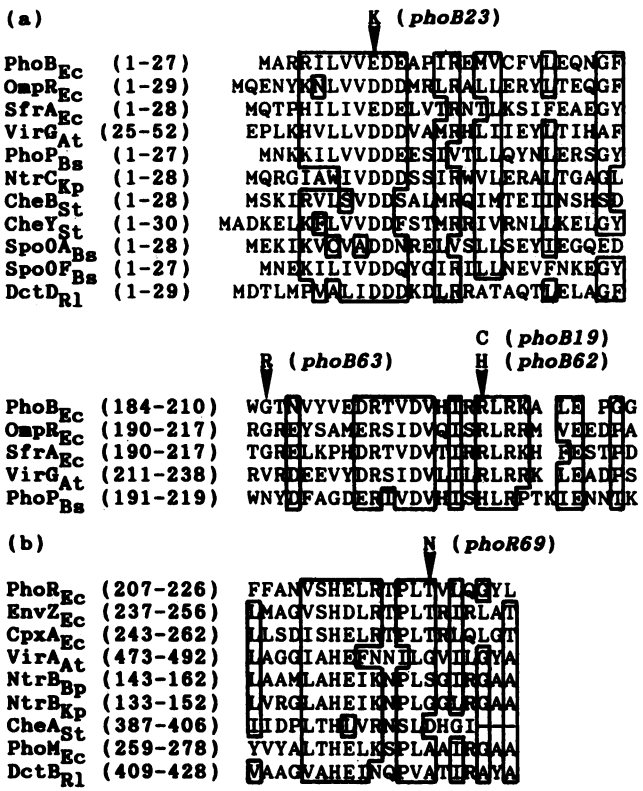

FIG. 3. Highly homologous regions conserved in the two-component regulatory proteins and the mutation sites in PhoB (a) or PhoR (b) proteins. Amino acid sequences of the regulatory proteins are from the published data (18, 23, 30, 38). Regions of proteins, indicated by the amino acid numbers in parentheses, have high homology with the corresponding regions of PhoB or PhoR proteins. Boxed residues are conserved, defined as more than 70% of amino acids belonging to one of the groups (A, G, P, S, T), (D, E, N, Q), (F, W, Y), (H, K, R), or (I, L, M, V). The amino acids substituted by mutation in PhoB or PhoR proteins are indicated by arrowheads together with the replaced amino acids. Abbreviations: At, *Agrobacterium tumefaciens*; Bp, *Bradyrhizobium parasponiae*; Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Rl, *Rhizobium leguminosarum*; St, *Salmonella typhimurium*.

severely, so this amino acid might be involved specifically in the protein folding in these three proteins rather than in the active sites, and the mutation might affect the stability of the protein.

The *phoR69* mutant is defective in repression of the *pho* regulon when there is an excess of phosphate, but retains inducing activity (35). This mutation had an alteration in Thr-220, where similar amino acids (Ala, Gly, Ser, and Thr) are conserved, to an amino acid belonging to a different group, Asn. The highly conserved region in this vicinity may be important for receiving environmental signals because the mutant is insensitive to the concentration of phosphate and therefore the PhoR69 protein is considered to activate PhoB constitutively.

The other *phoR* mutations (*phoR68*, *phoR17*, *phoR19*, *phoR20*, and *phoR78*) analyzed here were all nonsense mutations (Table 4), and therefore, prematurely terminated PhoR proteins are likely to be synthesized in the mutant strains in the absence of suppressor mutations. Since all of these mutants were null mutations defective in both the activator and inactivator functions for PhoB, the carboxyl-terminal half of PhoR may be essential for both functions. This result supports the notion that highly conserved regions among proteins with similar functions (in this case, the carboxyl-terminal half of PhoR) are functionally important.

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