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Bacillus subtilis PhoP Binds to the *phoB* Tandem Promoter Exclusively within the Phosphate Starvation-Inducible Promoter

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Several gene products, including three two-component systems, make up a signal transduction network that controls the phosphate starvation response in *Bacillus subtilis*. Epistasis experiments indicate that PhoP, a response regulator, is furthest downstream of the known regulators in the signaling pathway that regulates Pho regulon genes. We report the overexpression, purification, and use of PhoP in investigating its role in Pho regulon gene activation. PhoP was a substrate for both the kinase and phosphatase activities of its cognate sensor kinase, PhoR. It was not phosphorylated by acetyl phosphate. Purified phosphorylated PhoP (PhoP~P) had a half-life of approximately 2.5 h, which was reduced to about 15 min by addition of the same molar amount of *PhoR (the cytoplasmic region of PhoR). ATP significantly increased phosphatase activity of *PhoR on PhoP~P. In gel filtration and cross-linking studies, both PhoP and PhoP~P were shown to be dimers. The dimerization domain was located within the 135 amino acids at the N terminus of PhoP. Phosphorylated or unphosphorylated PhoP bound to one of the alkaline phosphatase gene promoters, the *phoB* promoter. Furthermore, PhoP bound exclusively to the −18 to −73 region (relative to the transcriptional start site +1) of the phosphate starvation-inducible promoter (P_v) but not to the adjacent developmentally regulated promoter (P_s). These data corroborate the genetic data for *phoB* regulation and suggest that activation of *phoB* is via direct interaction between PhoP and the *phoB* promoter. Studies of the phosphorylation, oligomerization, and DNA binding activity of the PhoP protein demonstrate that its N-terminal phosphorylation and dimerization domain and its C-terminal DNA binding domain function independently of one another, distinguishing PhoP from other response regulators, such as PhoB (*Escherichia coli*) and NtrC.

Bacillus subtilis responds to phosphate starvation by inducing Pho regulon gene transcription. This response is modulated by a regulatory network containing three two-component signal transduction systems (22, 60), two of which (ResDE and PhoPR) are involved in activating the Pho system (26, 27, 59) and one of which (Spo0A) functions to repress the Pho response (26). Epistasis experiments indicate that among the known Pho regulators, PhoP and PhoR are the furthest downstream and are essential for Pho regulon gene expression (22, 60). Parallel activation pathways upstream of PhoPR involve the response regulator ResD and a transition stage regulator, AbrB (22, 60). Transcription of the genes which encode these activators is repressed by phosphorylated Spo0A (Spo0A~P), thereby repressing the Pho response (26, 60).

The Pho regulon genes of *B. subtilis* include the structural genes for three secreted alkaline phosphatases (APases): *phoA* (encoding APase A), which is expressed primarily during phosphate starvation (20–23, 25, 26, 29); *phoB* (encoding APase B), which is expressed from tandem promoters either during phosphate starvation or during stage II of spore development (4, 9, 23, 25); and *phoD* (encoding APase D), which is expressed during phosphate starvation and encodes an enzyme with alkaline phosphodiesterase activity as well as APase activity (13). Other Pho genes include (i) the operon *tuaABCDEFGH*, which is responsible for synthesis of an anionic cell wall polymer, teichuronic acid, that replaces teichoic acid in the cell walls of phosphate-starved cells (58), (ii) the genes encoding the phosphate transport system, the *pstSACB1B2* operon (50), and (iii) the *phoPR* operon, encoding PhoP and PhoR (55, 56). Of the known Pho regulon promoters, the best characterized is the *phoB* promoter. The promoter region essential and sufficient for Pho regulation (P_v) has been separated from the upstream developmentally regulated promoter, P_s (9). Expression of the P_s promoter, but not the P_v promoter, is dependent on *spo0* genes and certain *spoII* genes (3, 9). Mutation in *phoP* or *phoR*, on the other hand, does not influence P_s expression but abolishes transcription of the P_v promoter (9, 22a). However, the mechanism through which PhoP and PhoR regulate the *phoB* promoter and any other Pho regulon genes, directly or indirectly, remains undefined.

PhoP and PhoR of *B. subtilis* have been considered homologs of PhoB and PhoR of *Escherichia coli* (55, 56), primarily because in either organism a null mutation in the gene encoding the protein with similarity to response regulators eliminates expression of reporter of the Pho response, APase(s). Both response regulators, PhoP (*B. subtilis*) and PhoB (*E. coli*), belong to the OmpR subfamily of response regulators, which has an H5-like DNA binding domain (35, 43, 61).

The *E. coli* PhoB and PhoR have been extensively studied. The cytoplasmic region of the *E. coli* PhoR, the sensor kinase, can be autophosphorylated by using ATP, and the phosphoryl group can be transferred from the truncated PhoR to PhoB, its response regulator (37). The unphosphorylated PhoB protein is a monomer in solution but dimerizes upon phosphorylation (16, 41). Phosphorylation has been found to increase the binding affinity of PhoB to the Pho box within the target gene promoters and activate gene transcription (19, 37, 38). Thus,

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Fiedler and Weiss proposed that a mutual functional inhibition between the N-terminal domain and the C-terminal domain exists in unphosphorylated PhoB (16). Phosphorylation of PhoB releases the functional inhibition between the two domains and leads to activation of the target genes through or by enhanced PhoB~P binding. Genetic evidence suggests that the concentration of the phosphorylated PhoB protein is regulated by *E. coli* PhoR acting as a kinase as well as a phosphatase (36, 65). The dual-functioning PhoR, together with the *pst* system and *phoU*, a negative regulator for phosphate metabolism, monitors the phosphate concentration in *E. coli* (63).

It has been postulated that PhoP-PhoR in *B. subtilis* regulates the phosphate response through a cascade of protein phosphorylation, as this is the major mechanism of two-component systems (20–22). Here, we provide evidence that PhoP acts as a response regulator for phosphate starvation regulation in *B. subtilis*. Our data suggest that PhoP is directly involved in Pho regulon gene activation. Biochemical studies of PhoP distinguish PhoP from other response regulators such as *E. coli* PhoB and enteric bacterial NtrC.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* DH5α was used as the host for plasmid constructions. *E. coli* BL21(DE3) (Novagen) served as the host for overexpressing the PhoP protein. The *phoP* gene was amplified from *B. subtilis* JH642 chromosomal DNA by PCR using primers FMH240 (5′-CAT^{198}ATGAACAAGAAA ATTTTAGT^{217}-3′) at the 5′ end of the gene and FMH144W (5′-AAATCGAT ^{927}CGGTATTTATTCATTC^{912}-3′) (32) at the 3′ end of the gene. The PCR product was cloned into pCRII (Invitrogen) to construct pWL31. The *phoP* gene was then released from pWL31 by *Nde*I and *Xho*I digestion and cloned into the *Nde*I and *Xho*I sites of pET16b (Novagen), yielding pWL32. As pWL32 contains a T7 *lac* promoter, the codons for 10 histidine residues, and an engineered Xa factor site upstream of the *phoP* gene, the overexpressed PhoP from this plasmid contained a His_{10} tag and another 11 amino acids at the N terminus (His_{10}-PhoP). Plasmid pWL32M was obtained in the same manner as pWL32 except that its *phoP* gene contains a nonsense mutation at amino acid 136. Thus, the overexpressed PhoP protein from this plasmid contains only the N-terminal 135 amino acids of the PhoP protein. The *phoP* genes in both pWL32 and pWL32M were confirmed by DNA sequencing. Plasmid pRC695 and pRC696 were used for making the *phoB* and P_v promoter probes (9).

Overexpression and purification of His_{10}-PhoP. *E. coli* BL21(DE3)pWL32 was incubated overnight at 37°C in LB medium containing carbenicillin (50 μg/ml) and was then inoculated into 2 liters of the same medium at a ratio of 1 to 100. The cells were grown at 30°C until the optical density at 600 nm (OD_{600}) of the culture reached 0.6. Then, 1 mM IPTG (isopropylthio-β-D-galactoside) was added to the culture, and growth was continued for another 3 h. The cells were harvested by centrifugation at 4°C and washed with sonication buffer (1 M NaCl, 5 mM $MgCl_2$, 50 mM Tris-Cl [pH 7.8]). The cell pellet was stored at −70°C.

The thawed cells were suspended on ice in 40 ml of sonication buffer containing 1 mM phenylmethysulfonyl fluoride and were immediately subjected to sonication. After a 4-min sonication on ice at an output of 100 W, the cell lysate was centrifuged at 120,000 × *g* for 1 h at 4°C. The supernatant fraction was filtered through a 0.45-μm-pore-size membrane and applied to a 1.5-ml nickel-nitrilotriacetic acid agarose (Qiagen) affinity column which was attached to a Waters 650E FPLC (fast protein liquid chromatography) system. The column was washed with sonication buffer until the OD_{280} of the eluate was less than 0.03; it was then washed with 30 mM imidazole in sonication buffer until the OD_{280} of the eluate was lower than 0.08. The protein on the column was eluted by using a gradient of 30 to 300 mM imidazole in sonication buffer. The protein peak fractions containing His_{10}-PhoP were pooled and dialyzed stepwise at 4°C against sonication buffer with decreasing concentrations of NaCl from 1 M to 0.8, 0.6, 0.4, 0.2, and finally 0.1 M. The protein was aliquoted and stored at −70°C. Before use, $CaCl_2$ (final concentration, 2 mM) was added, and the protein was digested with 1/50 (wt/wt) Xa factor (BioLabs) on a rocker for 5 h at room temperature. The resulting PhoP contained only one extra histidine at the N terminus and was used for all the experiments. For purification of the truncated PhoP protein, which has only the N-terminal 135 amino acids, a similar procedure was used, but the protein was eluted with 300 mM imidazole instead of by gradient on an FPLC system. This protein was also digested with Xa factor to produce N-PhoP.

Purification of *PhoR. The N terminus of the cytoplasmic region of the PhoR protein (from amino acids 226 to the end of the C terminus) was fused with GST (glutathione *S*-transferase). The recombinant protein was overexpressed in *E. coli* BL21 and purified as instructed by the manufacturer (Pharmacia). *PhoR, the cytoplasmic region of PhoR, was released by thrombin cleavage.

Phosphorylation of PhoP by PhoR. Two different forms of PhoR, prepared in phosphorylation buffer (50 mM KCl, 50 mM HEPES [pH 8.0], 5 mM $MgCl_2$), were used to phosphorylate PhoP. For the phosphotransfer test, gel shift assay, and DNase I footprinting experiments, *PhoR was used (see specific experiments for details).

For the experiments to determine the autophosphatase activity, stability, and oligomerization of PhoP~P, GST-*PhoR was used for the phosphorylation of PhoP. To test the stability of PhoP~P, 400 μl of boiled glutathione beads was washed with phosphorylation buffer and incubated with 500 μl of crude lysate of GST-*PhoR on a rocker at room temperature for 10 min. The unbound proteins were washed off the beads with 20 volumes of phosphorylation buffer, and the extra buffer was removed by microcentrifugation for 10 s. Then 60 μCi of [γ-^{32}P]ATP was added to the beads, and the autophosphorylation of GST-*PhoR was conducted at room temperature for 20 min. The beads were thoroughly washed with phosphorylation buffer until the flowthrough was free of ATP. The trace of ATP in the flowthrough was tested by adding *PhoR and measuring the formation of *PhoR~P. The failure to detect PhoR~P by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) indicated that the flowthrough was free of ATP. PhoP (40 μg) containing 5 mM dithiothreitol (DTT) and 50 mM KCl was added to the beads. After incubation at room temperature for 20 min, the PhoP~P was collected by centrifugation and passed through a Microcon 100 (Amicon) to remove any remaining beads. For the oligomerization of PhoP~P, GST-*PhoR on glutathione beads (see above) was incubated with 60 μCi of [γ-^{32}P]ATP, 400 μg of PhoP, 50 mM KCl, and 4 mM DTT at room temperature for 10 min. Then 5 mM cold (unlabeled) ATP was added, and the reaction was continued for another 20 min. The PhoP~P protein was separated from the GST-*PhoR as described above.

Phosphorylation of PhoP by acetyl phosphate. Radioactive ^{32}P-acetyl phosphate was prepared as described by Roggiani and Dubnau (51). PhoP (14 μM) or N-PhoP (14 μM) was incubated with 50 mM (final concentration) radioactive acetyl phosphate in 10 mM Tris-Cl (pH 7.0)–5 mM $MgCl_2$–4 mM DTT. The reaction was stopped by adding 1/5 volume of stop buffer (62.5 mM Tris-Cl [pH 6.8], 10% glycerol, 2% SDS, 5% 2-β-mercaptoethanol, 0.0012% bromophenol blue), and the samples were loaded onto gels for SDS-PAGE.

Detection of the stability of PhoP~P. To detect the autophosphatase activity of PhoP~P at room temperature, PhoP~P (3 μg) was added to 1/5 volume of stop buffer at different time points and loaded onto gels for SDS-PAGE. To determine the stability of PhoP~P under basic and acidic conditions, a final concentration of 0.1 N NaOH, 1 N NaOH, 0.1 N HCl, 1 N HCl, or H_2O (as a control) was incubated with PhoP~P individually at room temperature for 20 min before the samples were loaded for SDS-PAGE.

Dephosphorylation of *PhoP~P by PhoR. About 2 μM (1.8 μg) *PhoR, 2 μM (3.0 μg) bovine serum albumin (BSA), or phosphorylation buffer was added to 2 μM (1.2 μg) PhoP~P in the presence or absence of 1 mM ATP, and the reactions were conducted at room temperature for different periods of time. At each time point, the reaction was stopped by addition of 1/5 volume of stop buffer. The samples were subjected to SDS-PAGE, and the radioactivity was quantitated with a PhosphorImager (Molecular Dynamics, San Diego, Calif.).

Determination of oligomerization of PhoP, N-PhoP, and PhoP~P by gel filtration. PhoP, N-PhoP, and freshly prepared PhoP~P (see above) were individually loaded onto an HR10/30 Superdex 75 column (Pharmacia), preequilibrated with 50 mM Tris-HCl (pH 7.5)–100 mM KCl–4 mM DTT, and then the protein was eluted at 0.5 ml/min and collected in 400-μl fractions. The protein molecular weight standards (MW-GF-200 kit; Sigma) were applied to the column under the same conditions, and the elution volume and molecular weight of each standard were used to generate a standard curve to determine the molecular weight of the PhoP protein.

Protein cross-linking experiments. The *PhoP protein (0.8 μg, 0.13 μM) was mixed with 0.25 or 1.6 mM ethylene glycolbis(succinimidylsuccinate) (EGS; Pierce), and the cross-linking experiments were conducted in a 20-μl volume as described by Yang et al. (66) except that a final concentration of 50 mM Tris.Cl (pH 7.5) was used to terminate the reaction. After SDS-PAGE, the protein was detected by Western blotting using the PhoP antibody.

Gel shift assays. To make probes of the *phoB* and P_v promoters for gel shift assays, we used pRC695 and pRC696, respectively (9). The plasmids were digested with *Eco*RI separately and end labeled with Klenow fragment in the presence of [α-^{32}P]dATP. The inserts were released by *Bam*HI digestion. The probes were isolated as described by Manzara et al. (39). The DNA probe was further purified using an Elutip-d column (Schleicher & Schuell) as instructed by the manufacturer. In each reaction, *PhoR (1.4 μg) was incubated with 40 ng, 200 ng, 1 μg, or 5 μg of PhoP in the absence or presence of 5 mM ATP at room temperature for 15 min in binding buffer [50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 6.1), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 4 mM DTT, 4 mM $MgCl_2$]. The probe (5,000 cpm) was added to the reaction, and the incubation was continued for another 30 min. The samples were loaded onto a 6% native polyacrylamide gel made in 1× Tris-borate-EDTA (53). The gel was run at 4°C for 2 h, dried by vacuum, and exposed to an X-ray film.

DNase I footprinting of the *phoB* and P_v promoters. The noncoding strands of the two promoters were labeled as described above. To label the coding strands, pRC695 and pRC696 were digested by *Bam*HI and labeled with Klenow fragment in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. The plasmids were then digested by *Eco*RI to release the inserts. Purification of the probes was done as

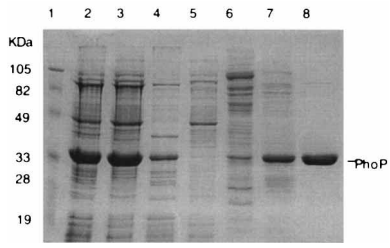

FIG. 1. Purification of overexpressed His_{10}-PhoP from *E. coli*. The fractions from each purification step were analyzed by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. The amount of protein loaded in each lane is given below in parentheses. Lanes: 1, prestained protein standards; 2, whole-cell sonication lysate (35 μg); 3, supernatant fraction (30 μg); 4, pellet fraction (5 μg); 5, flowthrough (4 μg); 6 and 7, two fractions from the 30 mM imidazole wash (5 and 8 μg, respectively); 8, eluate of His_{10}-PhoP by 30 to 300 mM imidazole gradient (12 μg).

described above. The same DNA binding reaction was used except that the amount of *PhoP was 40, 80, 160, or 320 ng and each reaction mixture contained ~20,000 cpm of probe. After the 30-min incubation at room temperature, 3 μl of DNase I (0.9 U) in 5 mM $MgCl_2$–5 mM KCl was added to each reaction mixture, and digestion was conducted for 40 s for the PhoP-containing samples and 20 s for *PhoP-free samples. The reactions were stopped, and the samples were prepared as described by Manzara et al. (39). The samples were run on a 6% polyacrylmide gel containing 7 M urea in 1× Tris-borate-EDTA for 1.5 h at 65 mA. The gel was then dried and exposed to an X-ray film with an intensifying screen at −70°C.

Detection of protein concentration. Protein concentration was detected by the Bradford method (7), using the Bio-Rad protein assay kit as instructed by the manufacturer.

SDS-PAGE and Western blot analysis. SDS-PAGE was done as described by Laemmli (31). A 12.5% separating gel was used for detection of His_{10}-PhoP or *PhoP, and a 15% separating gel was used for detection of N-*PhoP. The gel was either stained with Coomassie brilliant blue or dried and exposed to an X-ray film. The Western blotting was conducted as described previously (13), using either the PhoP antibody or the PhoP peptide antibody.

Antibody production. A 15-amino-acid peptide in PhoP (^{34}DGEEALKKA ETEKPD^{48}) (32) or the purified His_{10}-PhoP was used to inject into rabbits to generate PhoP antibody. The antibody was made by Cocalico Biologicals, Inc. (Reamstown, Pa.).

Quantitation of radioactivity. Radioactivity from gel filtration was measured with a scintillation counter (Packard, Meriden, Conn.). Radioactivity of proteins on SDS-PAGE was scanned and detected with a PhosphorImager (Molecular Dynamics). For calculation of DNA protection in footprinting gels, we followed the method described by Brenowitz et al. (8).

RESULTS

Overexpression and purification of His_{10}-PhoP. The His_{10}-PhoP protein was successfully overexpressed in *E. coli*, and it accounted for 30% of the total cellular protein (Fig. 1). More than 80% of the PhoP protein expressed at 30°C was in the soluble fraction after centrifugation of 120,000 × *g* for 1 h. The soluble His_{10}-PhoP which bound to a nickel-nitrilotriacetic acid affinity column showed different binding affinities: about 30% of the His_{10}-PhoP was eluted by a 30 mM imidazole wash, and 70% was eluted at 110 mM imidazole. The latter exhibited greater than 95% homogeneity, as determined by SDS-PAGE with Coomasie brilliant blue staining (Fig. 1), and represented a recovery of 20% of the total cellular proteins. After digestion with Xa factor, approximately 95% of the His_{10}-PhoP (Fig. 2, lane 7) was cleaved to produce PhoP (27 kDa) (Fig. 2, lane 6). The N-terminus sequencing indicated that Xa factor cut the fusion protein at the correct site (data not shown). Using Western blotting, we found that PhoP cross-reacted with the PhoP peptide antibody and the His_{10}-PhoP antibody (data not shown). A C-terminally truncated PhoP (15.4 kDa), N-PhoP, was also purified to homogeneity and cross-reacted with the His_{10}-PhoP antibody (data not shown).

Phosphorylation of PhoP by *PhoR. To determine if PhoP could be phosphorylated by PhoR, *PhoR was incubated with His_{10}-PhoP or PhoP in the presence of [γ-^{32}P]ATP. Figure 2 shows that both His_{10}-PhoP and PhoP could be phosphorylated by *PhoR (lanes 3 and 2, respectively). Unlike *PhoR, PhoP was not phosphorylated by ATP alone (Lane 4), suggesting that while *PhoR functions as an autophosphorylation kinase, PhoP is not autophosphorylated. N-PhoP could also be phosphorylated by *PhoR~P (data not shown), indicating that the phosphorylation domain of PhoP is at its N terminus as in other response regulators. The phosphotransfer between PhoP and *PhoR is rapid, reaching a steady state in less than 10 s at room temperature (56a).

It is not uncommon to see in vitro phosphotransfer between the histidine kinases and their noncognate response regulators (17, 46), although the phosphotransfer rate is at least 2 orders of magnitude lower than that for cognate pairs (58). However, we observed no phosphotransfer between the *E. coli* CheA-CheY and *B. subtilis* PhoR-PhoP when heterologous in vitro experiments were conducted (data not shown).

Many response regulators have been shown to be phosphorylated by low-molecular-weight phosphate donors, such as acetyl phosphate, phosphoramidate, and carbamyl phosphate (11, 34, 41). It has also been suggested that acetyl phosphate may play a role in the global regulation of *E. coli* (15, 40, 62). Both PhoP and N-PhoP were tested for the ability to use radioactive acetyl phosphate as a substrate. The positive control, *E. coli* CheY, was phosphorylated efficiently by acetyl phosphate, as reported by Lukat et al. (34). However, neither PhoP or N-PhoP could use acetyl phosphate as a substrate for phosphorylation (data not shown).

Stability of PhoP~P under acidic and basic conditions. To analyze the properties of PhoP~P with respect to the phosphorylated residue, the stability of PhoP~P under both acidic and basic conditions was tested. PhoP~P remained relatively stable in low pH conditions: after exposure to 0.1 N (pH 2.5) and 1 N HCl (pH 1) for 20 min at room temperature, the remaining radioactivities were 70 and 0.7%, respectively. In contrast, PhoP~P is labile under basic conditions: there was only 2% radioactivity left following treatment with 0.1 N NaOH (pH 11) and no radioactivity left after treatment with 1 N NaOH (pH 13) (data not shown). The relative acid/base stability of PhoP~P is consistent with the phosphorylation properties of an aspartate in other response regulators and suggests that an acyl phosphate is formed during phosphorylation of PhoP (42, 43).

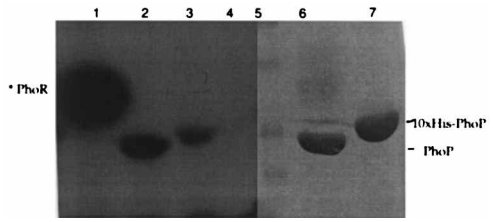

FIG. 2. Phosphorylation of His_{10}-PhoP and *PhoP by PhoR in the presence of ATP. Both His_{10}-PhoP (12.5 μg) and PhoP (10 μg) were incubated with 2.0 μg of *PhoR and 1 μCi of [γ-^{32}P]ATP at room temperature for 20 min. The samples were loaded for SDS-PAGE. The gel was dried and exposed to an X-ray film (lanes 1 to 4). Both His_{10}-PhoP and PhoP were also loaded for SDS-PAGE, and the gel was stained with Coommassie brilliant blue (lanes 5 to 7). Lanes: 1, *PhoR incubated with [γ-^{32}P]ATP; 2, PhoP incubated with *PhoR and [γ-^{32}P]ATP; 3, His_{10}-PhoP incubated with *PhoR and [γ-^{32}P]ATP; 4, PhoP incubated with only [γ-^{32}P]ATP; 5, prestained protein standards; 6, PhoP; 7, His_{10}-PhoP.

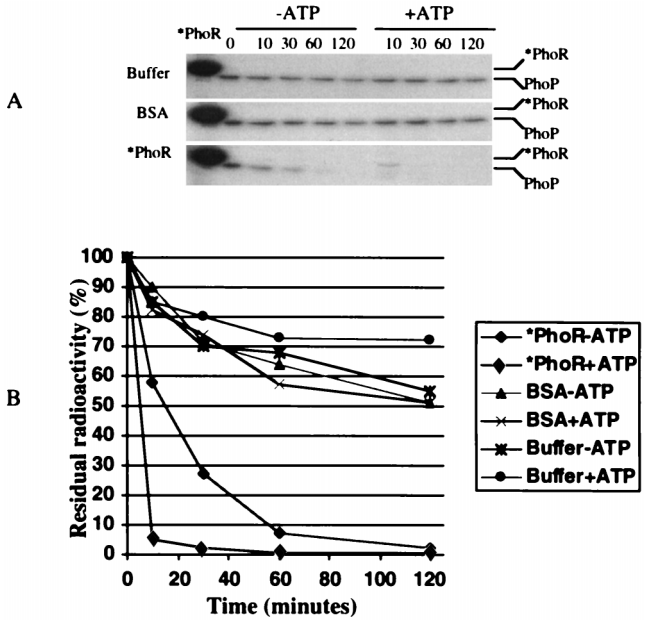

FIG. 3. Dephosphorylation of PhoP~P by *PhoR. Phosphorylated PhoP (1.2 μg, 2 μM) was incubated with *PhoR (1.8 μg, 2 μM), BSA (3 μg, 2 μM), or phosphorylation buffer in the presence or absence of 1 mM ATP for different periods of time before the reaction was stopped. The samples were subjected to SDS-PAGE, and the radioactivity was counted with a PhosphorImager. (A) Autoradiograph from SDS-PAGE. The numbers indicate incubation time in minutes. (B) Dephosphorylation curve of PhoP~P.

Dephosphorylation of PhoP~P by *PhoR. Certain histidine kinases exert an additional phosphatase activity on their phosphorylated response regulators (28, 33, 45). To test if PhoP~P can be dephosphorylated by *PhoR, PhoP was phosphorylated by GST-*PhoR and PhoP~P was isolated from GST-*PhoR (see Materials and Methods). PhoP~P was incubated with same molar ratio of *PhoR or BSA or with phosphorylation buffer without protein for different periods of time at room temperature (Fig. 3). When BSA or buffer was added, dephosphorylation of PhoP~P (due to autophosphatase activity) was rather slow, with a half-life of about 2.5 h. In contrast, in the presence of *PhoR, dephosphorylation of PhoP~P was increased. After 10 min, only about 60% (compared to 85%) of PhoP~P remained.

The phosphatase activity of OmpR and NtrB requires cofactors such as ATP (28, 30). To examine if dephosphorylation of PhoP~P by *PhoR is influenced by the presence of ATP, cold ATP (1 mM, final concentration) was added to the reactions discussed above. Addition of ATP in the reactions containing BSA or phosphorylation buffer did not apparently change the autophosphatase activity of PhoP~P. However, the presence of ATP stimulated phosphatase activity of *PhoR on PhoP~P dramatically. After 10 min of incubation, only 5% of PhoP~P was detected (Fig. 3).

It is noteworthy that a low level of *PhoR~P was detected when PhoP~P was incubated with *PhoR. This was pronounced in the presence of cold ATP. The phosphorylation of *PhoR was not due to autophosphorylation of *PhoR by radioactive ATP, because (i) no *PhoR~P was detected when *PhoR was incubated in the flowthrough from the washed glutathione beads, which were used for making PhoP~P (data not shown); (ii) the concentration of cold ATP added into the reaction represented at least 1,000-fold molar excess compared to PhoP~P. Our data suggest that the radioactivity in *PhoR was from PhoP~P. With increase of incubation time, *PhoR~P was decreased while substrate PhoP~P was reduced.

Oligomerization of PhoP under unphosphorylated and phosphorylated conditions. Certain response regulators, such as *E. coli* PhoB (16, 41), NtrC (16), and *B. subtilis* Spo0A (1), oligomerize upon phosphorylation. We used gel filtration to determine the oligomerization states of PhoP and PhoP~P. Purified PhoP was considered unphosphorylated, as the conditions for overexpression of the protein did not favor the formation of PhoP~P. PhoP, N-PhoP, and PhoP phosphorylated by GST-*PhoR were individually applied to a Superdex 75 gel filtration column. Unphosphorylated PhoP was eluted at 63 kDa and thus appeared as a dimer (Fig. 4A). When N-PhoP was loaded onto the column, it was also eluted as a dimer (Fig. 4A), indicating that the dimerization domain of PhoP is at the N terminus. The elution pattern of phosphorylated PhoP from the column showed that the protein peak and the major radio-

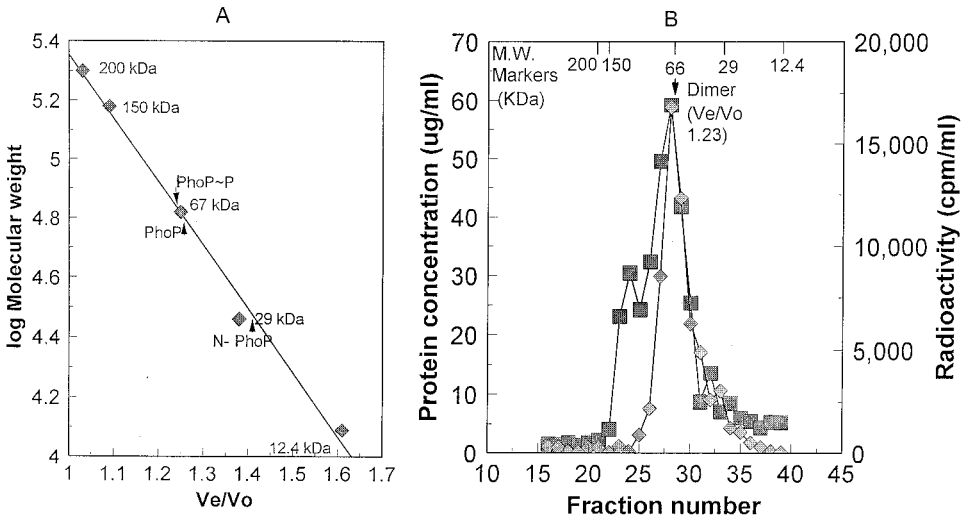

FIG. 4. Oligomeric states of PhoP, N-PhoP, and PhoP~P detected by gel filtration. (A) The protein molecular weight standard curve, on which the elution positions of PhoP, N-PhoP, and PhoP~P are indicated with arrows. ◆, molecular weight of standard. (B) Profile of phosphorylated PhoP on a Superdex 75 column. ◆, protein concentration; ■, radioactivity. M.W., molecular weight.

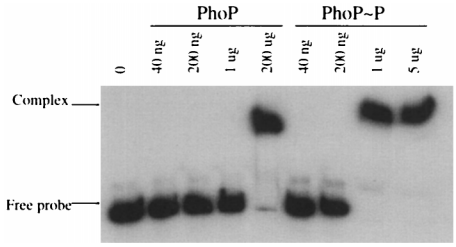

FIG. 5. Gel shift assay of the *phoB* promoter by PhoP and PhoP~P. PhoP (40 ng, 200 ng, 1 μg, and 5 μg) was incubated with *PhoR in the presence or absence of ATP. The 197-bp *phoB* promoter probe was added. After the binding reaction, the samples were loaded to a 5% native polyacrylamide gel to separate the free DNA and the DNA-protein complex. The amount of PhoP protein added to the reaction is indicated above each lane.

activity peak were coincident at one position (Fig. 4B) and identified as PhoP~P by SDS-PAGE and autoradiography (data not shown), indicating that PhoP~P is a dimer. A second radioactive peak was eluted at approximately 120 kDa; this did not contain the PhoP protein but contained a high-molecular-weight contaminant protein (probably GST-*PhoR dimer).

To confirm that *PhoP forms a dimer in solution, EGS was used to treat PhoP. Under our testing conditions, a PhoP dimer created by a covalent bond was detected after EGS treatment, but no dimer was observed in the untreated sample (data not shown). The formation of this cross-linking dimer supports the gel filtration data.

Binding of the *phoB* and P_v promoters by PhoP and PhoP~P. To determine if PhoP binds to the Pho regulon promoters, PhoP and PhoP~P were incubated separately with the *phoB* or P_v promoter for gel shift assays. The P_v promoter is one of the two tandem promoters in the *phoB* promoter and expressed only under phosphate starvation conditions (9). With increasing PhoP concentration, we observed that both *phoB* and P_v promoters mobility decreased in the presence or absence of ATP (all the reactions contained *PhoR), suggesting that both unphosphorylated PhoP and phosphorylated PhoP bind to the two promoters (Fig. 5 and data not shown). However, less protein was needed to observe retardation of the *phoB* promoter when PhoP was phosphorylated, indicating that PhoP~P binds to the *phoB* promoter more efficiently.

To locate the PhoP binding site on the *phoB* and P_v promoters, DNase I footprinting was performed on both strands of the *phoB* and P_v promoters. The results in Fig. 6A and B show that large regions of the *phoB* and P_v promoters on both strands were protected by PhoP at −6 to −65 (coding strand) and −18 to −73 (noncoding strand). The PhoP binding region of the P_v promoter was the same as in the *phoB* promoter on the coding strand. Protection of the noncoding strand in the P_v promoter seemed not as broad as that in the *phoB* promoter, probably because the protection site is too close to the labeled end. It is noteworthy that PhoP bound to the *phoB* promoter only in the P_v promoter region, which is responsible for vegetative APase production, not in the P_s promoter region, which expresses only during sporulation (Fig. 7). Several hypersensitive sites were observed in the binding region of the *phoB* and P_v coding strand in the presence of either PhoP or PhoP~P. However, these sites became more obvious when PhoP~P was used (Fig. 6A and B).

In the footprinting assay, we observed that higher percentage of DNA was bound by PhoP~P when same amounts of PhoP~P and PhoP were used (Fig. 6C). This was the case for both the P_v promoter and the *phoB* promoter.

DISCUSSION

Among the known regulators controlling the phosphate starvation response in *B. subtilis*, genetic evidence has shown that PhoP is responsible for the last step in the signaling pathway (22, 60). To determine if the activation of Pho regulon genes by PhoP involves direct promoter binding and to explore the mechanism through which PhoP regulates the Pho regulon genes, we used biochemical tools to characterize the PhoP protein with respect to phosphorylation, oligomerization, and DNA binding activity. The two proteins used in this study are PhoP, a PhoP protein with one extra histidine at its N terminus, and N-PhoP, a truncated PhoP protein containing the N-terminal 135 amino acids of PhoP.

It has been proposed that the level of phosphorylated response regulators in the cell is important for either activation or the cessation of activation of their target genes (52). Many response regulators have high autophosphatase activities resulting in a short half-life, which enables them to terminate the response once the signal dissipates. In contrast, the half-life of the active form of PhoP, PhoP~P, was approximately 2.5 h, indicating that PhoP has relatively weak autophosphatase activity. Expression of Pho regulon genes is repressed when phosphate-starved cells gain access to phosphate concentrations of greater than 0.1 mM (24); thus, a second dephosphorylation mechanism must exist to compensate for the weak self-dephosphorylation activity of PhoP. Dephosphorylation of PhoP~P by PhoR could be the answer since under in vitro conditions, PhoR reduced the half-life of PhoP~P from 2.5 h to about 15 min and the phosphatase activity of *PhoR was significantly increased in the presence of ATP. The autophosphatase activity of PhoP and phosphatase activity of PhoR are similar to those of *Rhizobium meliloti* FixJ and FixL, respectively: both FixJ~P and PhoP~P have relatively long half-lives (4 and 2.5 h, respectively); both *PhoR and truncated cytoplasmic FixL have an ATP-independent phosphatase activity (34). However, ATP was found to significantly stimulate the phosphatase activity of PhoR but not that of FixL (34). Since the phosphatases for response regulators can be enzymes other than their cognate kinases (18, 47, 48), we cannot exclude the possibility that there are phosphatases for PhoP~P in addition to PhoR.

Reverse phosphotransfer was observed from PhoP to PhoR (Fig. 3). The phosphorylated residue in PhoR~P resulting from this reverse phosphotransfer reaction is not known. The phosphoryl group may be covalently linked to the same histidine residue used in autophosphorylation, a result reported for an EnvZ mutant (12), or to other residues such as a serine residue as in the case of the APase reaction (14, 54). Whether *PhoR~P is an intermediate in the phosphatase or phosphotransferase reaction is not clear.

The activation of response regulators by phosphorylation is believed to be indirect (6). For both NtrC and PhoB, it has been suggested that the N-terminal domain and C-terminal domain inhibit each other's oligomerization and DNA binding functions (16). In these cases, phosphorylation of the response regulator is believed to release this mutual functional inhibition, allowing the N terminus to dimerize or oligomerize and the C terminus to bind to the target DNA (16). The crystal structure of *E. coli* NarL supports this idea, showing that in the unphosphorylated state, the C-terminal DNA binding domain folds back onto the N terminus and is not accessible to the DNA, implying that the C terminus is inhibited by the N terminus (2). In this study, PhoP dimerized and bound to the *phoB* promoter regardless of its phosphorylation state. Our data suggest that PhoP is a member of another class of re-

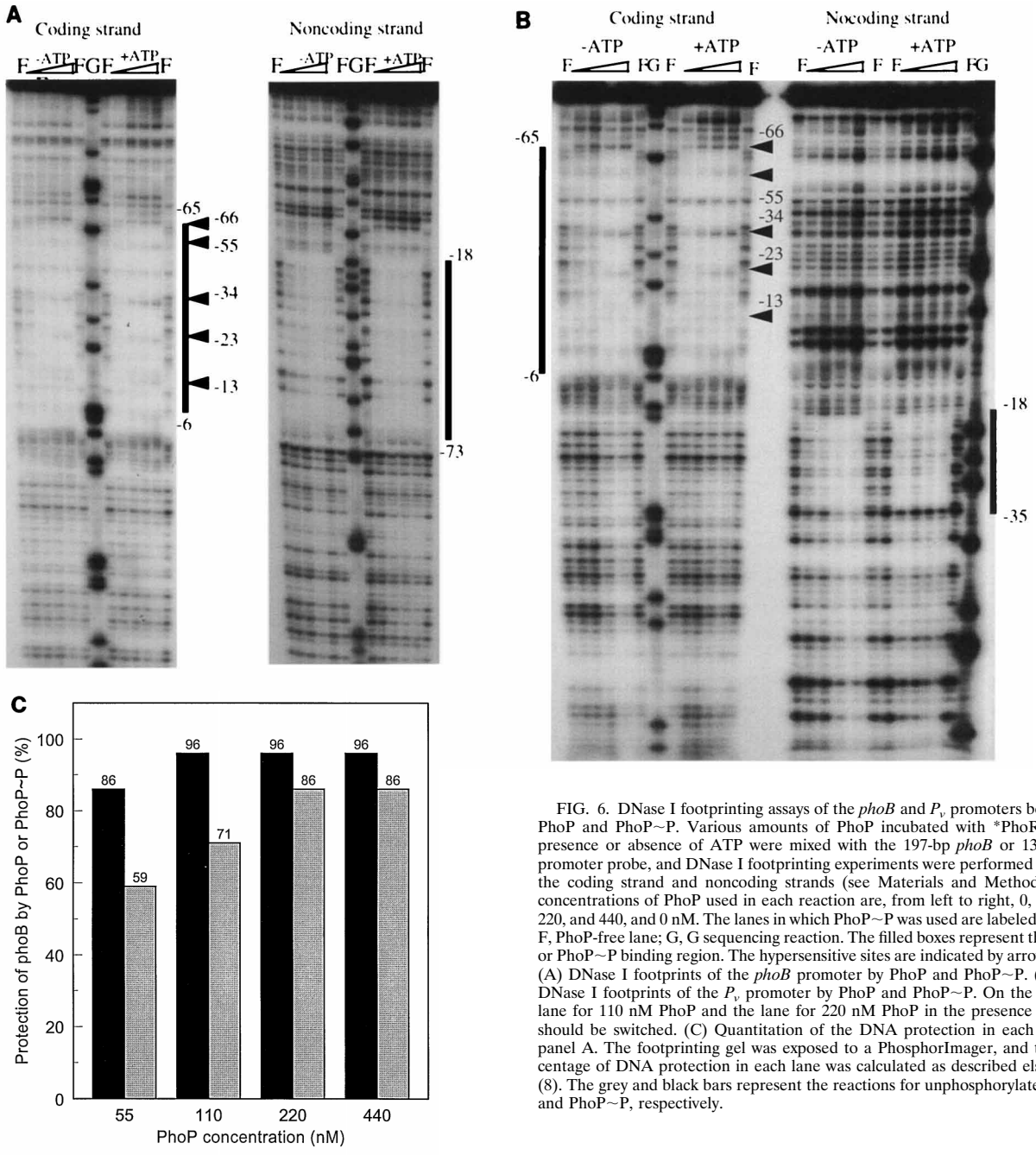

FIG. 6. DNase I footprinting assays of the *phoB* and P_v promoters bound by PhoP and PhoP~P. Various amounts of PhoP incubated with *PhoR in the presence or absence of ATP were mixed with the 197-bp *phoB* or 137-bp P_v promoter probe, and DNase I footprinting experiments were performed on both the coding strand and noncoding strands (see Materials and Methods). The concentrations of PhoP used in each reaction are, from left to right, 0, 55, 110, 220, and 440, and 0 nM. The lanes in which PhoP~P was used are labeled +ATP. F, PhoP-free lane; G, G sequencing reaction. The filled boxes represent the PhoP or PhoP~P binding region. The hypersensitive sites are indicated by arrowheads. (A) DNase I footprints of the *phoB* promoter by PhoP and PhoP~P. (B) The DNase I footprints of the P_v promoter by PhoP and PhoP~P. On the gel, the lane for 110 nM PhoP and the lane for 220 nM PhoP in the presence of ATP should be switched. (C) Quantitation of the DNA protection in each lane of panel A. The footprinting gel was exposed to a PhosphorImager, and the percentage of DNA protection in each lane was calculated as described elsewhere (8). The grey and black bars represent the reactions for unphosphorylated PhoP and PhoP~P, respectively.

sponse regulators in which the N terminus and the C terminus function independently. Similar results have recently been reported for *E. coli* UhpA and *Bordetella pertussis* BvgA (5, 10). In the case of UhpA, phosphorylation does not change the oligomeric state of the monomeric protein (10). For this class of proteins, phosphorylation may function only to change the conformation of proteins, resulting in transcriptional activation.

Although less phosphorylated PhoP was needed to achieve the same degree of protection within the *phoB* and P_v promoters, the difference in binding affinity was not dramatic as that for unphosphorylated PhoP. We propose that binding of the phosphorylated PhoP to the target promoters may involve protein-protein interactions in addition to DNA-protein interactions as suggested for *E. coli* OmpR and NtrC (44, 64). Therefore, phosphorylation may change the protein oligomerization as well as DNA binding affinity. Under our conditions, we did not observe a difference in the oligomerization of the phosphorylated and unphosphorylated PhoP protein in solution. However, a higher than dimer oligomeric state may form in the presence of target DNA. In enteric bacteria, formation of large oligomers at the enhancer binding site is required for activation of gene transcription. The formation of these large oligomers is facilitated by phosphorylation (49). Some of these oligomers bind to DNA, and some others formed via protein-protein interaction build up on DNA but not along DNA (64). Other response regulators, including *B. subtilis* PhoP, may uti-

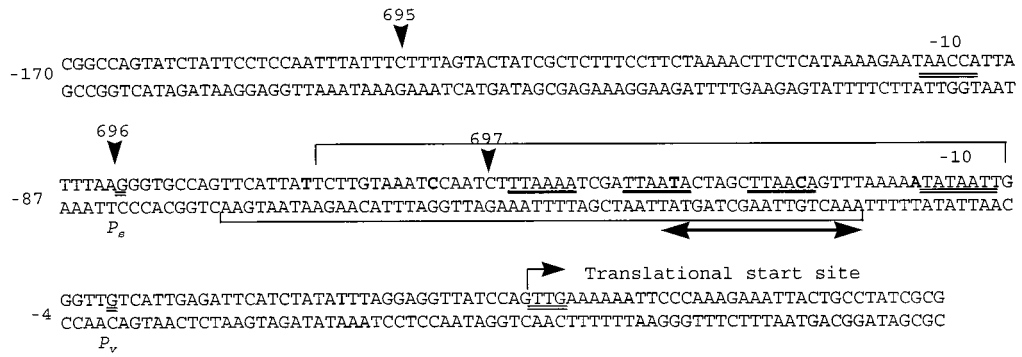

FIG. 7. PhoP and PhoP~P binding sites on the two strands of the *phoB* promoter region. The solid lines above and below the sequences represent the PhoP or PhoP~P binding region on the coding strand and noncoding strands, respectively. The underlines show the conserved 6-bp TTAACA-like sequence in the *phoB* promoter. The transcription start sites for both P_v and P_s, the −10 sequence of the two promoters, and the translation start site (TTG) are double underlined. Arrowheads labeled 695, 696, and 697 represent the start of the 5′ in the promoter deletions made previously for examining the promoter activity (9). The double-headed arrow represents the PhoP binding region in the noncoding strand of the P_v promoter. The hypersensitive sites are in boldface.

lize a similar mechanism involving large-oligomer formation to activate transcription.

B. subtilis has an APase multigene family. *phoB*, encoding APase B, contributes 25% of total APase specific activity during phosphate starvation induction and 50% of the APase specific activity expressed during induction at stage II of sporulation (4). Deletion analysis identified the promoter region responsible for *phoB* expression under each condition (9). The observation that PhoP binding to the complete *phoB* promoter was identical with and confined to the P_v promoter is consistent with the conclusions that during phosphate starvation, the *phoB* promoter activity (Fig. 7, construct 695) is entirely from P_v (Fig. 7, construct 696) and that this promoter activity is dependent on PhoP (9). That PhoP did not bind to the P_s promoter region of *phoB*, which is located upstream of the P_v promoter, corroborates the fact that PhoP is not required for sporulation APase expression (22a). A partial 5′ deletion of the PhoP binding site identified within the P_v promoter (Fig. 7, construct 697) totally abolishes P_v transcription during phosphate starvation (9), underscoring the importance of PhoP binding for activation of the P_v promoter. Within the PhoP binding region of the P_v promoter, there are three tandem TTAACA-like sequences separated by four to five nucleotides (Fig. 7). A similar sequence is observed in the *B. subtilis pstS* promoter, suggesting that this region may be the *B. subtilis* Pho box (50). The hypersensitive sites in the coding strand of the *phoB* and P_v promoters were observed only in the presence of PhoP or PhoP~P and were located on the same face of the DNA double helix (appearing every 11 or 22 bp), indicating that PhoP bent the promoter in the same direction. It has been reported that *E. coli* PhoB binds to the *E. coli* Pho box in the major groove and bends the promoter in the same direction to bring the PhoB protein and σ^{70} close to each other in order to activate transcription (35). The binding and bending of the *phoB* promoters by PhoP may be the common mechanism by which PhoP activates transcription of the *B. subtilis* Pho regulon genes.

In conclusion, we demonstrate that the N terminus and the C terminus of the unphosphorylated PhoP protein do not mutually inhibit their domain functions, in contrast to NtrC and *E. coli* PhoB. Based on the data presented in this report, we envision that transcriptional activation of the Pho regulon genes occurs by the direct interaction of PhoP~P, the RNA polymerase, and the Pho regulon promoters. Although the different roles of unphosphorylated PhoP and phosphorylated PhoP in transcriptional activation are not known, the ongoing studies of in vitro transcription of the Pho regulon genes by using both forms of the PhoP protein will test their function in Pho regulon gene transcription.

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