Target Genes and DNA-Binding Sites of the Response Regulator PhoR from *Corynebacterium glutamicum*[†]

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The two-component signal transduction system PhoRS of *Corynebacterium glutamicum* is involved in the phosphate (P_i) starvation response. To analyze the binding of unphosphorylated and phosphorylated PhoR to the promoters of phosphate starvation-inducible (*psi*) genes, this response regulator and the kinase domain of its cognate sensor, PhoS (MBP-PhoSΔ1-246), were overproduced and purified. MBP-PhoSΔ1-246 showed constitutive autophosphorylation activity, and a rapid phosphoryl group transfer from phosphorylated MBP-PhoSΔ1-246 to PhoR was observed. Gel mobility shift assays revealed that phosphorylation increases the DNA-binding affinity of PhoR. The affinity of PhoR~P to different promoters varied and decreased in the order *pstSCAB* > *phoRS* > *phoC* > *ushA* > *porB* > *ugpA* > *pitA* > *nucH* and *phoH1* > *glpQ1*. The binding sites in front of *pstSCAB* and *phoRS* were localized at positions –194 to –176 and –61 to –43 upstream of the transcriptional start sites, respectively. Alignment of these two 19-bp binding sites revealed a high identity in the 5'-terminal part, but not in the 3'-terminal part. As many OmpR-type response regulators bind to direct repeats, the 19-bp sequence might be interpreted as a loosely conserved 8-bp direct repeat separated by 3 bp. This idea was supported by the fact that the highest binding affinity was observed with a perfect 8-bp direct repeat of the sequence CCTGTGAAatCCTGTGAA. Inspection of the other target promoters revealed sequences with some similarity to this binding motif, which might represent PhoR binding sites. The *in vivo* relevance of the PhoR-binding site within the *phoRS* promoter was supported by reporter gene studies.

Phosphorus is an essential nutrient for all cells and is required, e.g., for the biosynthesis of nucleotides, DNA, and RNA, but also for the regulation of protein activity by phosphorylation of histidine, aspartate, serine, threonine, or tyrosine residues. A common phosphorus source is inorganic phosphate (P_i), and cells have developed mechanisms for the acquisition, assimilation, and storage of phosphate. When P_i becomes limiting, many bacteria induce the synthesis of proteins that enable them to capture the residual P_i resources more efficiently and to make alternative phosphorus sources accessible. The corresponding genes are collectively named P_i starvation-inducible genes, or *psi* genes. The phosphate starvation response, particularly its regulation, has been most carefully studied in *Escherichia coli* (28) and *Bacillus subtilis* (12).

We recently started to characterize the phosphate starvation response in *Corynebacterium glutamicum* (13), a gram-positive soil bacterium used industrially for the production of more than 2 million tons of amino acids per year, mainly L-glutamate and L-lysine (11). An overview of the biology, genetics, physiology, and application of *C. glutamicum* can be found in a recent monograph (5). Phosphorus constitutes 1.5% to 2.1% of the cell dry weight of *C. glutamicum* (18), part of which is present as polyphosphate (15, 17, 21). The phosphate starvation stimulon of *C. glutamicum* was determined using whole-genome DNA microarrays (13). Comparison of the mRNA profiles before and at different times after a shift from P_i excess

to P_i starvation led to the identification of a group of genes that are presumably required to cope with a limited P_i supply. This group includes the *pstSCAB* operon, encoding an ABC transporter for high-affinity P_i uptake; the *ugpAEBC* operon, encoding an ABC transporter for uptake of glycerol 3-phosphate; *glpQ1*, encoding a glycerophosphoryl diester phosphodiesterase; *ushA*, encoding a secreted enzyme with UDP sugar hydrolase and 5'-nucleotidase activity (22); *nucH*, encoding a putative secreted nuclease that possibly plays a role in liberating P_i from extracellular nucleic acids; *phoC* (NCgl2959/cg3393), which may encode a cell wall-associated phosphatase (29); *phoH1*, encoding an ATPase of unknown function; and the *pctABCD* operon, encoding an ABC transport system that might be involved in the uptake of a yet-unknown phosphorus-containing compound (13).

In most bacteria analyzed in this respect, the phosphate starvation response is controlled by two-component signal transduction systems, e.g., the PhoBR system in *E. coli* (28) or the PhoPR system in *B. subtilis* (12). When we screened a set of 12 *C. glutamicum* mutants, each lacking the genes for one two-component system, one of the strains showed a growth defect under P_i starvation compared to the wild type (16). The genes lacking in this strain were named *phoS* and *phoR* and encode a histidine kinase and a response regulator, respectively. The role of the PhoRS system in the adaptation to P_i starvation was supported by DNA microarray studies that showed that the *psi* genes, except those of the *pstSCAB* operon, were not induced in the Δ*phoRS* mutant within 1 hour after a shift from P_i excess to P_i limitation, in contrast to the wild type (16). In the study presented here, we analyzed whether the *C. glutamicum* response regulator PhoR is able to interact with the promoters of the *psi* genes, determined the influence of

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TABLE 1. Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Source/reference
<i>E. coli</i> DH5 α	F [−] <i>thi-1 endA1 hsdR17(r[−] m⁺) supE44 ΔlacU169 (φ80lacZΔM15) recA1 gyrA96 relA1</i> ; host for cloning procedures	Invitrogen
<i>E. coli</i> BL21(DE3)	F [−] <i>ompT hsdSB(r_B[−] m_B[−]) gal dcm</i> (λcIts857 <i>ind1</i> Sam7 <i>nin5 lacUV5-T7</i> gene 1); host for overproduction of plasmid-encoded recombinant proteins	Novagen
<i>C. glutamicum</i> ATCC 13032	Biotin-auxotrophic wild-type strain	1
Plasmids		
pET16b	Amp ^r ; <i>P_{T7} lacI oriV</i> from pBR322; <i>E. coli</i> expression vector for overproduction of proteins with an N-terminal decahistidine tag that can be cleaved off by factor Xa	Novagen
pET16b-PhoR	Amp ^r ; pET16b derivative for overproduction of PhoR with an N-terminal decahistidine tag (PhoR _{N-His10})	This work
pMal-c	Amp ^r ; <i>P_{lac} lacI^q ColE1 oriV</i> ; <i>E. coli</i> expression vector for construction and overproduction of fusion proteins containing the MBP of <i>E. coli</i> (MalE) without its signal peptide	New England Biolabs
pMBP-PhoSΔ1-246	Amp ^r ; pMal-c derivative for overproduction of the kinase domain of <i>C. glutamicum</i> PhoS (amino acid residues 247 to 485) fused to the C-terminus of the <i>E. coli</i> MBP without signal peptide (MBP-PhoSΔ1-246)	This work
pET2	Kan ^r ; promoter-probe vector	27
pET2-phoR1	Kan ^r ; pET2 with a 233-bp fragment of the <i>phoR</i> promoter	This work
pET2-phoR2	Kan ^r ; pET2 with a 200-bp fragment of the <i>phoR</i> promoter	This work
pET2-phoR3	Kan ^r ; pET2 with a 180-bp fragment of the <i>phoR</i> promoter	This work

phosphorylation on the DNA-binding affinity, and characterized the binding sites in front of *pstSCAB* and *phoRS* in detail.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains and plasmids used or constructed in this work are listed in Table 1. *E. coli* DH5 α (Invitrogen) was used as a host during the construction of recombinant plasmids and was routinely grown aerobically at 37°C on a rotary shaker (120 rpm) in Luria-Bertani (LB) medium (23). *E. coli* BL21(DE3) (26) was used for overproduction of the proteins MBP-PhoSΔ1-246 and PhoR and was grown aerobically at 30°C on a rotary shaker (120 rpm) in LB medium. When appropriate, ampicillin was added at a concentration of 100 μg/ml. The *C. glutamicum* strain ATCC 13032 (1) was used as the wild type. For chloramphenicol-acetyltransferase (CAT) assays, 5 ml of brain heart infusion (BHI) medium (Difco) was inoculated with colonies from a fresh BHI agar plate and incubated for about 8 h at 30°C and 170 rpm. The cells of this first preculture were used to inoculate a 500-ml shake flask containing 50 ml of CGXII minimal medium with 4% (wt/vol) glucose (14) and 30 mg/liter 3,4-dihydroxybenzoic acid as an iron chelator. The second preculture was incubated overnight at 30°C and 120 rpm and then used to inoculate the main culture (300 ml of the CGXII medium described above in a 1-liter shake flask) to an optical density at 600 nm (OD₆₀₀) of 1. This culture was incubated at 30°C and 120 rpm until an OD₆₀₀ of 4 to 5 was reached. At that time, cells from 30 ml culture were harvested by centrifugation on ice and stored at −20°C until they were used. Two hundred milliliters of the remaining culture was harvested, washed twice in CGXII medium lacking phosphate and glucose, resuspended in 200 ml CGXII-glucose medium without phosphate, and incubated as described above. Ten, 30, 60, 90, and 120 min after the transfer to P_i-free medium, cells from 30 ml culture were harvested and stored at −20°C until they were used. For growth of *C. glutamicum* harboring pET2-phoR plasmids, the medium was supplemented with 25 μg/ml kanamycin.

General DNA techniques and sequence analyses. The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany) or New England Biolabs (Frankfurt, Germany). The oligonucleotides used in this study were obtained from Operon (Cologne, Germany) and are listed in Table S1 in the supplemental material. Routine methods, like PCR, DNA restriction, or DNA ligation, were carried out according to standard protocols (23). Chromosomal DNA from *C. glutamicum* was prepared as described previously (6). Plasmids from *E. coli* were isolated with the QIAprep spin miniprep kit (QIAGEN). *E. coli* was transformed by the RbCl method (9). DNA sequencing was performed with a Genetic Analyzer 3100-Avant (Applied Biosystems). Sequencing reactions were carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Construction of expression plasmids pMBP-PhoSΔ1-246 and pET16b-PhoR. For overproduction and purification of the kinase domain of the *C. glutamicum* sensor kinase PhoS, the expression plasmid pMBP-PhoSΔ1-246 was constructed. The DNA region encoding the kinase domain (amino acids 247 to 485) was amplified by PCR using primers that introduced an EcoRI restriction site before codon 247 (primer *phoS*Δ1-246-EcoRI-fw) and a PstI restriction site after the stop codon (primer *phoS*Δ1-246-PstI-rv). The resulting PCR product was cloned into pMal-c (New England Biolabs, Frankfurt, Germany) cut with EcoRI and PstI, and the relevant portion of the resulting plasmid, pMBP-PhoSΔ1-246, was checked for the absence of spurious mutations by DNA sequencing. The cloning strategy resulted in a protein (MBP-PhoSΔ1-246; 624 amino acids; 67.7 kDa) in which the kinase domain of PhoS was fused to the carboxy terminus of the *E. coli* maltose binding protein (MBP) lacking its signal peptide.

For overproduction and purification of the response regulator PhoR with an N-terminal cleavable decahistidine tag, the expression plasmid pET16b-PhoR was constructed. The *phoR* coding region was amplified using primers that introduced an NdeI restriction site overlapping the start codon (primer *phoR*-NdeI-fw) and an XhoI restriction site after the stop codon (primer *phoR*-XhoI-rv). After digestion with NdeI and XhoI, the 767-bp PCR product was cloned into the expression vector pET16b (Novagen). The PCR-derived part of the resulting pET16b-PhoR plasmid and the ligation sites were sequenced in order to exclude unwanted mutations. The PhoR_{N-His10} protein encoded by this plasmid contained 21 additional amino acids (MGHHHHHHHHHHSSGHHIEGRH) at the amino terminus, including a factor Xa cleavage site (SGHHIEGR). After cleavage of PhoR_{N-His10} with factor Xa, the resulting PhoR protein contained only one additional amino acid (H) at the amino terminus.

Overproduction and purification of PhoR and MBP-PhoSΔ1-246. For overproduction of PhoR and MBP-PhoSΔ1-246, *E. coli* BL21(DE3) transformed with one of the expression plasmids was cultivated in LB medium up to an OD₆₀₀ of 0.3. Then, expression of the target gene was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the culture was incubated for another 3 h at room temperature. Cells were harvested, washed once in TNGI5 buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, 5% [vol/vol] glycerol, and 5 mM imidazole; the number indicates the millimolar imidazole concentration), and resuspended in the same buffer (~1.5 g [wet weight] in 6 ml buffer) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 mM diisopropylfluorophosphate. After disruption using a French pressure cell, intact cells and cell debris were removed by centrifugation (20 min at 5,000 × g; 4°C), and the supernatant was subjected to ultracentrifugation (1 h at 150,000 × g; 4°C). The resulting supernatant was passed through a 0.2-μm filter and used for protein purification.

MBP-PhoSΔ1-246 was purified by affinity chromatography on amylose resin (New England Biolabs) equilibrated with TNGI5 buffer. After being washed with 30 ml TNGI5 buffer, MBP-PhoSΔ1-246 was eluted with 10 1-ml portions TNGI5 buffer containing 10 mM maltose. Fractions containing MBP-PhoSΔ1-246 were

FIG. 1. Coomassie-stained SDS-polyacrylamide gels showing overproduction and purification of PhoR_{N-His10} and MBP-PhoSΔ1-246. Additionally, the successful cleavage of the N-terminal His tag of PhoR_{N-His10} by factor Xa is shown. Lanes 1, 4, 7, and 10, protein standards (Precision Plus Protein Prestained Standard; Bio-Rad); lanes 2 and 3, whole-cell lysates of *E. coli* BL21(DE3)/pET16b-PhoR before and 3 h after IPTG induction, respectively; lane 5, 1.3 μg of purified PhoR_{N-His10}; lane 6, 1.3 μg of PhoR after factor Xa digestion of PhoR_{N-His10}; lanes 8 and 9, whole-cell lysates of *E. coli* BL21(DE3)/pMBP-PhoSΔ1-246 before and 3 h after IPTG induction, respectively; lane 11, 3 μg of purified MBP-PhoSΔ1-246.

pooled, the buffer was exchanged for GMS buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, and 10% [vol/vol] glycerol) using PD-10 columns (GE Healthcare), and the purified protein was stored at -20°C.

PhoR_{N-His10} was purified by Ni²⁺ chelate affinity chromatography using His-bind resin (Novagen) equilibrated with TNGI5 buffer. After being washed using buffers with increasing imidazole concentrations (20 ml TNGI5, 15 ml TNGI20, 15 ml TNGI50, and 10 ml TNGI70), PhoR_{N-His10} was eluted with eight 1-ml portions TNGI200 buffer. Fractions containing PhoR_{N-His10} were pooled, and the buffer was exchanged for GMS buffer using PD-10 columns (GE Healthcare). The purified protein was stored at -20°C. As initial gel mobility shift assays revealed that the His tag decreased the DNA-binding affinity, the tag was routinely cleaved off using factor Xa (Novagen). Briefly, purified PhoR_{N-His10} was digested for about 14 h at 20°C with factor Xa (20 U/mg PhoR_{N-His10}), and subsequently, factor Xa was removed with Xarrest agarose. The purified PhoR was stored at -20°C. Overproduction and purification of MBP-PhoSΔ1-246 and PhoR, as well as the proteolytic His tag cleavage, were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining of the proteins with Coomassie brilliant blue.

In vitro phosphorylation assays. To determine its autophosphorylation activity, 12 μM MBP-PhoSΔ1-246 was incubated with 0.17 μM [γ -³²P]ATP (10 mCi/ml; GE Healthcare) and 80 μM nonradioactive ATP. The assay mixture was incubated at room temperature, and at different time points, aliquots were removed, mixed with an equal volume of 2× SDS loading buffer (124 mM Tris-HCl, pH 6.8, 20% glycerol, 4.6% SDS, 1.4 M β-mercaptoethanol, 0.01% bromophenol blue), and placed on ice. Subsequently, without prior heating, the samples were subjected to SDS-PAGE using a 12% separating gel. After being dried, the gel was analyzed with a BAS-1800 phosphorimager (Fujifilm). For analyzing phosphorylation of PhoR by MBP-PhoSΔ1-246, a twofold molar excess of purified PhoR was added to the MBP-PhoSΔ1-246-containing assay mixture described above (after it had been incubated for 5 min), and the sample was incubated for another 61 min. At different time points, aliquots were removed (taking into consideration the changed MBP-PhoSΔ1-246 concentration) and handled as described above.

Gel mobility shift assays. For gel mobility shift assays, a variety of different DNA fragments (89 to 704 bp) generated by PCR and purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) were mixed with PhoR_{N-His10} or with PhoR, either in the unphosphorylated state or after phosphorylation with MBP-PhoSΔ1-246. The reaction mixture included ~100 ng target DNA (10 to 55 nM), a 0- to 300-fold molar excess of protein, and GMS buffer in a total volume of 20 μl. For phosphorylation of PhoR, it was incubated for 60 min with MBP-PhoSΔ1-246 (half the concentration of PhoR) and 5 mM ATP before the DNA fragments were added. In general, the protein-DNA mixtures were incubated for 30 min at room temperature and then separated on a 15% native polyacrylamide gel. Electrophoresis and staining were performed as described previously (30) but using precooled electrophoresis buffer.

For testing the effect of mutations in PhoR-binding sites, the desired mutated DNA fragments were constructed by overlap extension PCR. For this purpose, a 22-bp region harboring the presumed binding site was placed between two fragments of the *pstS* promoter that do not bind PhoR. These two flanking

FIG. 2. Autophosphorylation of MBP-PhoSΔ1-246 and phosphoryl group transfer from MBP-PhoSΔ1-246~P to PhoR. MBP-PhoSΔ1-246 (12 μM) was incubated in TKMD buffer (50 mM Tris-HCl [pH 7.5], 200 mM KCl, 5 mM MgCl₂, and 5 mM dithiothreitol) at room temperature with 0.17 μM [γ -³²P]ATP (~3,000 Ci/mmol; 10 mCi/ml) and 80 μM nonradioactive ATP in a total volume of 60 μl; 1 min (lane 1) and 5 min (lane 2) after ATP addition, 7-μl samples were removed. After 5 min, 30 μl of a 25.8 μM PhoR solution was added to 30 μl of the remaining MBP-PhoSΔ1-246 solution, and 14-μl samples were taken after 1 min (lane 3), 6 min (lane 4), 21 min (lane 5), and 61 min (lane 6). All samples were mixed immediately with 2× SDS loading buffer and stored on ice. Subsequently, without prior heating, the samples were subjected to SDS-PAGE using a 12% separating gel. After being dried, the gel was analyzed with a BAS-1800 phosphorimager (Fujifilm).

regions extend from positions -72 to +162 (flanking region 1) and from positions -369 to -283 (flanking region 2) relative to the transcriptional start site (+1) determined previously (16). For amplification of flanking region 1, primer P_{pstS rev}, covering nucleotides +162 to +140 of the *pstS* promoter, was used in combination with a 49-mer oligonucleotide. The 5'-terminal 22 nucleotides of this 49-mer covered the PhoR-binding site, including the desired mutations, and the 3'-terminal 27 nucleotides corresponded to positions -72 to -45 of the *pstS* promoter. For amplification of flanking region 2, primer P_{pstS fw}, covering nucleotides -369 to -347 of the *pstS* promoter, was used in combination with a second 49-mer oligonucleotide. Its 5'-terminal 22 nucleotides covered the complementary PhoR-binding site, including the desired mutations, and its 3'-terminal 27 nucleotides corresponded to nucleotides -283 to -310 of the *pstS* promoter. The PCR fragments obtained with these two primer pairs were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and 50 ng of each fragment was used as a template for overlap extension PCR with the primers P_{pstS fw} and P_{pstS rev}. The resulting PCR product was purified as described above and used in the gel mobility shift assays.

Construction of transcriptional fusions and CAT assays. For CAT assays, the full-length *phoR* promoter (fragment phoR1) and two *phoR* promoter subfragments (fragments phoR2 and phoR3) were amplified and cloned into the corynebacterial promoter-probe vector pET2 (27), resulting in plasmids pET2-phoR1, pET2-phoR2, and pET2-phoR3. The cloned fragments were controlled by DNA sequence analysis. The plasmids were introduced into wild-type *C. glutamicum*, and the transformed strains were cultivated as outlined above. The CAT assays were performed as described previously (7).

RESULTS

Autophosphorylation of MBP-PhoSΔ1-246 and phosphoryl group transfer to the response regulator PhoR. To demonstrate the phosphorylation activities that are characteristic of two-component signal transduction systems, the kinase domain of PhoS (MBP-PhoSΔ1-246) and the entire PhoR protein were overproduced in *E. coli* and purified (Fig. 1). To determine whether MBP-PhoSΔ1-246 possesses autophosphorylation activity, the protein was incubated with [γ -³²P]ATP, and samples taken at different times were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 2, MBP-PhoSΔ1-246 catalyzed its own phosphorylation, reaching a maximal phosphorylation level after about 5 min of incubation (data not shown). When purified PhoR was added at this time, an efficient transfer of the phosphoryl group from MBP-PhoSΔ1-246 to PhoR

FIG. 3. Comparison of the DNA-binding affinities of unphosphorylated and phosphorylated PhoR to the promoter regions of *pstS* and *nucH*. Fragments covering the promoter regions of *nucH* (10 nM), *pstS* (10 nM), and the control genes *gpmA* (15 nM) and *acn* (27 nM) were incubated for 30 min at room temperature with increasing concentrations of PhoR (A) or PhoR~P (B). Then, the 20- μ l reaction mixtures were separated by electrophoresis on 15% native polyacrylamide gels to separate free DNA and DNA-protein complexes, and the gels were stained with SybrGreen I. For phosphorylation of PhoR, the protein was preincubated for 60 min at room temperature with a twofold-lower concentration of MBP-PhoSΔ1-246 and 5 mM ATP.

was observed within 1 minute (Fig. 2). Maximal PhoR phosphorylation levels were reached after 30 to 60 min. No phosphorylation of PhoR was observed when the protein was incubated with [γ - 32 P]ATP for 60 min in the absence of MBP-PhoSΔ1-246 (data not shown).

Binding of PhoR to putative target promoters and influence of phosphorylation on the binding affinity. According to our previous data, rapid induction of the *psi* genes after a shift from P_i excess to P_i starvation is dependent on the PhoRS two-component system (16). We therefore analyzed whether the response regulator PhoR can bind to the promoter regions of the *psi* genes. In a first experiment, the promoter regions of the *pstSCAB* operon and of *nucH*, together with those of *acn* (aconitase) and *gpmA* (cg0482; phosphoglycerate mutase), which served as negative controls, were incubated with PhoR up to a 300-fold molar excess of protein. As shown in the upper part of Fig. 3, unphosphorylated PhoR was able to bind to the *pstS* promoter. About 50% of the *pst* promoter fragment was shifted at a PhoR concentration of 2.1 μ M. In contrast, PhoR did not bind to the *nucH* promoter or to the promoter regions used as controls. In a parallel experiment, PhoR was preincubated for 60 min with MBP-PhoSΔ1-246 and 5 mM ATP before addition of the DNA fragments. As shown in the lower part of Fig. 3, the phosphorylation of PhoR had a significant influence on its binding properties. In the case of the *pstS* promoter, the affinity was increased about fivefold, as about 50% of the *pstS* promoter fragment was shifted at a PhoR~P concentration of $\sim$ 0.45 μ M. In the case of the *nucH* promoter, about 50% of the fragment was shifted at the maximal PhoR~P concentration used in this experiment (3.15 μ M), whereas no shift was observed again with the two control fragments. This experiment showed that, as in the cases of *E. coli* PhoB (28) and *B. subtilis* PhoP (19), phosphorylation in-

creases the DNA-binding affinity of *C. glutamicum* PhoR. At the same time, the experiment indicates that PhoR~P has different binding affinities to different target promoters.

After it had been shown successfully that PhoR interacts with two of the predicted target promoters, binding of PhoR to the promoter regions of *pstSCAB*, *phoRS*, *phoC*, *ushA*, *ugpAEBC*, *nucH*, *phoH1*, *glpQ1*, and *pctABCD* was tested individually. In all these binding assays, the promoter region of the *acn* gene served as a negative control. As shown in Fig. 4, PhoP phosphorylated by MBP-PhoSΔ1-246 was able to bind to each of the promoter fragments tested; however, the binding affinities differed. They decreased in the order *pstS* ($\sim$ 35 $\times$) > *phoR* ($\sim$ 45 $\times$) > *phoC* ($\sim$ 50 $\times$) > *ushA* ($\sim$ 60 $\times$) > *ugpA* ($\sim$ 70 $\times$) > *nucH* and *phoH1* ($\sim$ 120 $\times$) > *glpQ1* ($>$ 120 $\times$). The values in parentheses roughly indicate the molar excess of PhoR~P required to shift 50% of the DNA fragment. In the case of the promoter region of the *pctABCD* operon, very weak binding was observed, requiring a 150- to 200-fold molar excess of PhoR~P to shift half of the DNA fragments (data not shown). The *acn* control promoter was not shifted in any of the experiments.

Another gene whose promoter region was tested for binding of PhoR was *porB*, which was described as an anion-specific channel or porin in the cell wall of *C. glutamicum* (3). The mRNA level of the *porB* gene was reproducibly reduced in the Δ *phoRS* mutant, under both P_i excess and P_i limitation (data not shown). As shown in Fig. 4, the *porB* promoter region was shifted by PhoR, requiring an $\sim$ 70-fold molar excess of PhoR~P for binding half of the DNA fragments. This suggests that PhoR might be an activator of *porB* expression, independent of P_i starvation conditions. As porins were recently reported to be required for P_i uptake in *Mycobacterium smegmatis* (31), PorB might have such a function in *C. glutamicum*.

Reinspection of our previous microarray data (13) revealed that the mRNA level of the *pitA* gene encoding a low-affinity phosphate uptake system (29) was reproducibly reduced in the wild type after a shift from P_i excess to P_i starvation, although the value did not match the arbitrarily chosen criteria (fourfold-changed mRNA level, on average) used to summarize the genes of the P_i starvation stimulon (see Table 2 in reference 13). Therefore, we also analyzed the binding of PhoR to the promoter region of *pitA*. As shown in Fig. 4, the *pitA* promoter fragment was indeed shifted, requiring a 90-fold molar excess of PhoR~P to bind half of the DNA fragments. PhoR might thus function as a repressor of the *pitA* gene.

In parallel to binding assays with phosphorylated PhoR, unphosphorylated PhoR was also tested. For all promoters analyzed, the binding affinity of unphosphorylated PhoR was much lower than that of the phosphorylated form or binding was even undetectable (Fig. 4B).

Delimitation of the PhoR-binding sites in the *pstS* and *phoR* promoter regions. Attempts to find a conserved sequence motif in the promoter regions of the putative PhoR target genes were not successful. Therefore, gel mobility shift assays with subfragments of the promoter regions of *pstS* and *phoR* were performed in order to confine the PhoR-binding sites in these promoters (Fig. 5). The promoters of *pstS* and *phoR* were chosen because they showed the highest affinity to PhoR in the preceding experiments. In the case of the *pstS* promoter, the binding assays suggested the presence of two binding regions,

FIG. 4. Binding of PhoR~P and PhoR to individual promoter regions. (A) DNA fragments (14 to 38 nM) covering the promoter regions of *pstS*, *phoR*, *phoC*, *ushA*, *porB*, *ugpA*, *pitA*, *nucH*, *phoH1*, *glpQ1*, and *acn* (negative control) were incubated for 30 min at room temperature with a 0- to 120-fold molar excess of PhoR~P. Subsequently, the reaction mixtures (20 μ l) were separated by electrophoresis on a native 15% polyacrylamide gel, and the gels were stained with SybrGreen. (B) The same DNA fragments used in panel A were incubated with a 0- to 300-fold molar excess of unphosphorylated PhoR and then treated as described above.

one located between -175 and -201 and another one between -99 and -136 relative to the transcriptional start site (Fig. 5). In the case of the *phoR* promoter, the most likely binding site was located between -40 and -65 relative to the transcriptional start site (Fig. 5). Comparison of the corresponding sequences still did not allow the identification of a common sequence motif.

Mutational analysis of the promoter-distal PhoR-binding site within the *pstS* promoter. Further gel mobility shift assays with DNA fragments containing the region from -175 to -201 of the *pstS* promoter confirmed the presence of a PhoR-binding site between -176 and -194 relative to the transcription start site, as the insertion of this 22-bp sequence between two fragments of 87 bp and 234 bp that are not shifted resulted in a 343-bp fragment that was shifted by PhoR with high affinity. To test which of the 22 bp are critical for PhoR binding, a mutational analysis was performed, and the results are summarized in Fig. 6A. In a first series of experiments, 3 or 4 bp were exchanged simultaneously (fragments 2 to 8). The mutations in fragments 3 and 8 strongly inhibited PhoR binding, and those in fragments 2, 4, 5, 6, and 7 led to a weaker decrease in the binding affinity. In a second series of experiments, each of

bp 4 to 22 was exchanged separately (fragments 9 to 27). The exchanges in fragments 9, 13, 15, 16, 19, and 25 had no obvious effect on PhoR binding; those in fragments 10, 14, 17, 23, and 24 decreased binding weakly; those in fragments 18, 22, 26, and 27 decreased binding more strongly; and those in fragments 11 and 12 decreased binding very strongly. Remarkably, the mutations in fragments 20 and 21 had a positive influence on the binding affinity. In a third series of experiments, the base pairs whose exchange had the strongest negative influence on PhoR binding were combined (fragments 28 to 30). As expected, an additive effect was observed: exchange of only 2 of the 22 bp was sufficient to prevent PhoR binding almost completely (fragments 29 and 30). Thus, specific nucleotides that are of prime importance for PhoR binding could be determined.

Mutational analysis of the PhoR-binding site within the *phoR* promoter. Further analysis of the *phoR* promoter region led to the identification of a putative PhoR-binding site located between -43 and -61 with respect to the transcriptional start site. This 19-bp region was subjected to the same kind of mutational analysis described above, and the results are depicted in Fig. 6B. When 3 or 4 bp were exchanged simultaneously, five of the resulting fragments (fragments 2, 3, 4, 6,

FIG. 5. Search for PhoR-binding sites within the promoter regions of *pstS* and *phoR*. Gel mobility shift assays were performed with subfragments (14 to 55 nM) of these promoter regions and PhoR~P (for details, see the legend to Fig. 3). The numbers indicate the ends of the fragments relative to the transcription start site (+1). Based on the observed shifts, the fragments were divided into five categories: +++, 50% of the fragment shifted with a <30-fold molar excess of PhoR~P; ++, 50% of the fragment shifted with an ~30-fold molar excess of PhoR~P; +, 50% of the fragment shifted with a >30- to 60-fold molar excess of PhoR~P; (+), 20 to 50% of the fragment shifted at a >60- to 90-fold molar excess of PhoR~P; and -, 0 to 20% of the fragment shifted at a 120-fold molar excess of PhoR~P. Based on these results, two putative PhoR-binding sites were located in the *pstS* promoter (-99 to -136 and -175 to -201) and one in the *phoR* promoter region (-40 to -65).

A:	shift	B:	shift
1) AAACCTGTGAGAATAATAAGAA	+++	1) ACTGTGAATGTACTGAAAG	+++
2) CCC-----	++	2) CAG-----	(+)
3) ---AAG-----	(+)	3) ---TGT-----	(+)
4) ---TGT-----	+	4) ---CCG-----	(+)
5) ---CTC-----	++	5) ---TGC-----	+++
6) ---CGC-----	+	6) ---AGT-----	(+)
7) ---CTCC-----	(+)	7) ---CCCT-----	(+)
9) ---A-----	+++	8) C-----	++++
10) ---A-----	++	9) -A-----	+
11) ---G-----	(+)	10) -G-----	(+)
12) ---T-----	+	11) ---T-----	(+)
13) ---G-----	+++	12) ---G-----	+
14) ---T-----	++	13) ---T-----	+
15) ---C-----	+++	14) ---C-----	+
16) ---T-----	+++	15) ---C-----	+
17) ---C-----	++	16) ---G-----	++
18) ---C-----	++	17) ---T-----	++++
19) ---G-----	+++	18) ---G-----	++
20) ---C-----	++++	19) ---C-----	++
21) ---C-----	++++	20) ---A-----	+
22) ---G-----	+	21) ---G-----	(+)
23) ---C-----	++	22) ---T-----	+
24) ---C-----	++	23) ---C-----	(+)
25) ---T-----	+++	24) ---C-----	+
26) ---C-----	+	25) ---C-----	+
27) ---AAG-----CTCC	+	26) ---T-----	+
28) ---AAG-----CTCC	(+)		
29) ---G-----C	(+)		
30) ---G-----C	(+)		

FIG. 6. Mutational analysis of the PhoR-binding sites within the *pstS* promoter (A) and the *phoR* promoter (B). Previous experiments had indicated that fragments 1 have a high affinity to PhoR~P. To determine the relevance of individual base pairs for binding, fragments with different mutations were tested in gel mobility shift assays for the ability to bind PhoR~P (for details, see the legend to Fig. 3). According to the results of the shift (see Fig. S1 in the supplemental material), the fragments were divided into five categories: +++++, 50% of the fragment shifted with a <30-fold molar excess of PhoR~P; +++, 50% of the fragment shifted with an ~30-fold molar excess of PhoR~P; ++, 50% of the fragment shifted with a >30- to 45-fold molar excess of PhoR~P and 100% of the fragment shifted at a 60-fold molar excess of PhoR~P; +, <50% of the fragment shifted with a >30- to 45-fold molar excess of PhoR~P and 100% shifted at a 90-fold molar excess of PhoR~P; and (+), 50% of the fragment shifted at a >45- to 90-fold molar excess of PhoR~P.

and 7) showed a strongly reduced binding affinity and one (fragment 5) behaved like the parent fragment. These effects were reflected by the results obtained with fragments containing single exchanges (fragments 8 to 26). All mutations except two reduced the PhoR-binding affinity. The exchanges in fragments 8 and 17 led to an increased PhoR binding affinity.

Deduction and verification of a putative consensus binding site for PhoR. An alignment of the PhoR-binding sites within the *pstS* promoter and the *phoR* promoter (Fig. 7, no. 1 and 2) showed that 11 of the 19 bp were identical in the two sequences. Most prominent was the sequence CTGTGA in the 5' half of the 19-bp motif. One may interpret the binding sites as two very loosely conserved 8-bp direct repeats separated by a 3-bp spacer. To follow up this idea, binding of PhoR~P to a fragment containing the 8-bp sequence ACTGTGAA twice separated by the 3-bp spacer AAT and mutated derivatives of this sequence were tested in gel mobility shift assays. As shown in Fig. 8, the fragment containing this artificial sequence showed the highest affinity for PhoR of all fragments tested hitherto, and this affinity could still be improved by changing the 5'-terminal A residues of the 8-bp repeats to C residues (fragment 3). In this case, a 26-fold molar excess of PhoR was sufficient for a complete shift. The other mutations within the 8-bp repeat decreased the binding affinity, in the case of fragment 5 almost completely. When the 3-bp spacer region between the two 8-bp repeats was shortened to either 2 or 1 bp (fragments 6 and 7), binding was also almost completely prevented. The same was true for an increase of the spacer region to 6 bp (fragment 8). Similarly, there was essentially no binding of PhoR if either the 5'-terminal or the 3'-terminal 8-bp repeat

No.	Gene	Sequence (5' → 3')	Position	
1	<i>pstS</i>	CCTGTGAGAATAATAAGAA	-194 to -176	TS
2	<i>phoR</i>	ACTGTGAATGTACTGAAAG	-43 to -61	TS
3	motif	CCTGTGAANNCCCTGTGAA	see Fig. 8, lane 3	
4	<i>pstS</i>	TTTGTGATTAGAGTCTCCA	-135 to -117	TS
5	<i>pstS</i>	CATTGTGAGACTCTAATCA	-113 to -131	TS
6	<i>phoC</i>	AATGTGAACGCCTTAAAC	-159 to -141	TL
7	<i>ushA</i>	TGGGTGAACAGTACATAAA	-80 to -62	TS
8	<i>porB</i>	TATGTGAATGCTCTACTAA	-147 to -165	TL
9	<i>ugpA</i>	ACTGGGAGCAGGCTTCCAA	-148 to -130	TS
10	<i>pitA</i>	TTGGGGCTATAGCTATTAT	-6 to -24	TS
11	<i>nucH</i>	CCTATTAGCTGACCTTTAT	-90 to -108	TS
12	<i>phoH1</i>	ACTAGCAATGGTTTGTGGG	-242 to -260	TL
13	<i>phoH1</i>	AGTGTGAAGCATATGAACC	-132 to -114	TL
14	<i>glpQ1</i>	TCACTGAATCCGCTGCGGA	-248 to -266	TL
15	<i>glpQ1</i>	CCGTTGAAGCAGCTGTGCG	-203 to -221	TL

FIG. 7. Alignment of verified and putative PhoR binding sites. Binding sites 1 and 2 were identified in this work, and binding site 3 is an artificial binding site with the currently highest affinity for PhoR~P. It represents an 8-bp direct repeat separated by 3 bp. Sequences 4 to 15 were identified by visual inspection of the promoter regions to which PhoR binds *in vitro*. Positions are relative either to the transcriptional start site (TS) or, if this is not known, to the proposed translational start site (TL).

was completely exchanged for C residues. These data support the idea that the PhoR-binding site represents a degenerated 8-bp repeat separated by 3 bp and reveal CCTGTGAANN CCTGTGAA as currently the best binding site.

In vivo relevance of the PhoR-binding site in front of *phoRS*. In order to determine whether the PhoR-binding site within the *phoRS* promoter region is necessary for transcriptional activation of *phoRS* *in vivo*, the expression of three transcriptional fusions with a reporter gene encoding CAT in *C. glutamicum* was measured. The *phoR* promoter fragments that were cloned in front of the promoterless *cat* gene in plasmid pET2 (27) are shown in Fig. 9A. Plasmid pET2-phoR1 contained the entire *phoR* promoter region, including the PhoR-binding site; plasmid pET2-phoR2 lacked the PhoR-binding site but still contained the -10 and -35 regions of the promoter; and plasmid pET2-phoR3 also lacked these regions. The strains carrying these plasmids were cultivated in regular

CGXII minimal medium with 4% (wt/vol) glucose until mid-exponential phase (OD_{600} , ~5). Then, the cells were harvested, washed two times with CGXII without phosphate and glucose, and resuspended in CGXII glucose medium without phosphate. Immediately before harvest and 10, 30, 60, 90, and 120 min after the transfer to P_i -free medium, the CAT activities of cell extracts were determined. As shown in Fig. 9B, the strain with plasmid pET2-phoR1 grown under P_i excess showed a specific activity of about 300 mU/mg. Under P_i limitation, the specific activity increased to about 500 mU/mg within 60 min. The strain carrying plasmid pET2-phoR2 showed a threefold-lower CAT activity under P_i excess (100 mU/mg), and this activity remained constant under P_i limitation (Fig. 9C). The strain with plasmid pET2-phoR3 showed negligible CAT activity (<2 mU/mg) (Fig. 9D). It is not yet clear whether the threefold-lower CAT activity of the strain carrying pET2-phoR2 was due to an incomplete -35 region or to the fact that PhoR also activates *phoRS* expression to some extent under P_i excess. Nevertheless, the fact that there was no increase of *phoRS* expression under P_i limitation in the absence of the PhoR-binding site supports the relevance of this site for induction of the *phoRS* operon.

DISCUSSION

In our preceding study, we presented evidence that the PhoS-PhoR two-component signal transduction system of *C. glutamicum* is involved in the response to phosphate starvation. A Δ *phoRS* mutant showed impaired growth under P_i limitation, presumably because many of the genes required to cope with P_i starvation were not induced as rapidly and strongly as in the wild type (16). In the current study, we provide *in vitro* evidence for a direct regulation of the *psi* genes by PhoS-PhoR, as purified PhoR was able to bind to nine of the presumed target promoters. In many cases, response regulators that function as transcriptional activators and/or repressors show an increased DNA-binding affinity after phosphorylation, as reported, e.g., for CitB of *Klebsiella pneumoniae* (20) or NarL of *E. coli* (32). The isolated kinase domain of PhoS, which showed constitutive autophosphorylation activity, was used as a tool for the rapid *in vitro* phosphorylation of PhoR (Fig. 2). Phosphorylation led to an approximately fivefold increase of PhoR's DNA-binding affinity under our experimental conditions (Fig. 3 and 4). The factor of five is presumably a minimal value, as we do not know the percentage of PhoR that was actually phosphorylated in our binding assays, and is comparable to

FIG. 8. Experimental analysis of the putative PhoR consensus binding motif. Based on sequences 1 and 2 in Fig. 7, an artificial 19-bp consensus sequence consisting of two direct 8-bp repeats of the sequence ACTGTGAA and the spacer AAT was inserted between two fragments that were not bound by PhoR~P. The resulting fragment and mutated derivatives were tested in gel mobility shift assays for the ability to bind PhoR~P (for details, see the legends to Fig. 3 and 4).

FIG. 9. Test for the in vivo relevance of the proposed PhoR-binding site within the *phoR* promoter with plasmid-borne *phoR-cat* transcriptional fusions using *C. glutamicum* ATCC 13032 as a host. (A) Overview of the three *phoR* promoter fragments that were cloned in front of the promoterless *cat* gene in plasmid pET2. Fragment *phoR1* (-75 to +158 relative to the transcriptional start site) covers the entire *phoR* promoter region, including the PhoR-binding site (BS; -61 to -43) and the -10 and -35 regions; fragment *phoR2* (-42 to +158) lacks the PhoR-binding site but contains the -10 and -35 regions; and fragment *phoR3* (+22 to +158) also lacks the -10 and -35 regions. (B to D) Expression of the transcriptional fusions in *C. glutamicum* ATCC 13032 was determined by measuring the CAT activities of cells after cultivation in CGXII glucose minimal medium without phosphate for 0 to 120 min. The values for each fusion represent averages from measurements of three independent cultures. The error bars represent standard deviations.

that observed for other response regulators, e.g., PhoP of *B. subtilis*, whose DNA-binding affinity is increased about 10-fold by phosphorylation (19).

PhoR was shown to bind to eight promoters of *psi* genes/operons, i.e., *pstSCAB* (~35×) > *phoRS* (~45×) > *phoC* (~50×) > *ushA* (~60×) > *ugpAEBC* (~70×) > *nucH* and *phoH1* (~120×) > *glpQ1* (>120×), but with significantly different affinities, as indicated by the values in parentheses, which denote the molar excess of PhoR~P required to shift ~50% of the DNA fragment. There is a correlation, although not strict, between the binding affinity and the induction kinetics of the *psi* genes after a shift from P_i excess to P_i starvation (13), with genes having a high affinity for PhoR being more rapidly induced than genes with a low affinity. Thus, the induction kinetics of the individual *psi* genes may in part be determined by the binding affinities of their promoters for PhoR~P. The temporal pattern of induction of the different *psi* genes might allow the cells to adapt as efficiently and economically as possible to P_i limitation. The first response is the induction of the *pstSCAB* and *phoRS* genes, whose expression is increased up to eightfold within 10 min after a P_i downshift (13) and whose promoters show the highest affinity to PhoR (Fig. 4). The high-affinity ABC-type P_i uptake system encoded by *pstSCAB* allows the cell to scavenge those low P_i concentrations in the environment that cannot be taken up by low-affinity P_i transporters, e.g., PitA. The *psi* genes that are induced later than *pstSCAB* and *phoRS* and whose promoters have a lower affinity for PhoR encode transporters for the uptake of organophosphates, such as glycerol-3-phosphate, or extracytoplasmic enzymes, like PhoC, UshA (22), or NucH, that cleave phosphate from organophosphates and thus make it accessible for import by the PstSCAB system.

The promoter with the second-best affinity to PhoR was that of the *phoRS* operon itself (Fig. 4), supporting previous data that suggested a positive autoregulation of *phoRS* (16). Such a

type of regulation is a common feature of many two-component systems and can have different functions (2). In the case of the PhoS-PhoR system, elevated PhoR levels formed through autoinduction could be necessary to control all of the putative target promoters (≥10, according to our results) and at the same time could be responsible for the apparently hierarchical organization of the regulon. Whereas uninduced levels of PhoR~P are sufficient to induce the high-affinity *pstSCAB* and *phoRS* promoters, elevated levels of PhoR~P might be required for induction of the lower-affinity target promoters. Thus, the PhoS-PhoR system may function as a rheostat rather than a simple switch (2). This kind of behavior was previously also described for the BvgA-BvgS system of *Bordetella pertussis* (4, 24).

Using mutational analysis, it could be shown that PhoR binds to 19-bp regions within the *pstS* and the *phoR* promoter (Fig. 6). An alignment of these two sequences (Fig. 7, no. 1 and 2) revealed that in particular the 5'-terminal half was very similar. OmpR-type regulators with a winged helix-turn-helix DNA-binding motif, to which PhoR belongs, commonly bind to direct-repeat sequences, although there is high variability in the nucleotide sequences, the spacing between repeat units, and the number of repeat units. Based on this trait, one might interpret the PhoR-binding sites as two very loosely conserved 8-bp direct repeats separated by a 3-bp spacer. A similar binding motif was recently described for the response regulator MprA of *Mycobacterium tuberculosis* (10). Support for the assumption that PhoR binds to a direct repeat was obtained by the finding that an artificial 19-bp binding site composed of two 8-bp direct repeats of the 5'-terminal half of the *pstSCAB* and *phoRS* binding sites showed a higher affinity for PhoR~P than the natural binding motif with the highest affinity, i.e., that of the *pst* promoter. Moreover, reduction of the spacer between the two repeats from 3 to 2 bp almost completely prevented PhoR~P binding. As the distance of 11 bp between

the centers of the two repeats corresponds approximately to one full turn of the DNA helix, this spacing would allow the binding of two PhoR monomers in a side-by-side manner on the same face of the DNA helix. Gel filtration studies with unphosphorylated PhoR indicated an apparent molecular mass between 33 and 37 kDa (data not shown). Since PhoR (calculated mass, 26.5 kDa) is composed of two domains that are connected by a flexible linker (the receiver domain and the DNA-binding domain), its migration in gel filtration might be faster than that of the globular proteins used for calibration. Therefore, an apparent mass of ~35 kDa is compatible with a monomeric form of PhoR. Gel filtration studies with PhoR that had been phosphorylated by preincubation with MBP-PhoSΔ1-246 and ATP led to the same results. However, as we do not know the percentage of PhoR that becomes phosphorylated in this way, definite conclusions as to the influence of phosphorylation on the oligomerization status of PhoR are not yet possible. If phosphorylated PhoR is also monomeric, binding of one monomer to an 8-bp repeat unit might favor the binding of a second monomer to the neighboring 8-bp repeat (cooperative binding). In the case of PhoP from *M. tuberculosis*, the response regulator with the highest sequence identity (65%) to *C. glutamicum* PhoR in this *Mycobacterium* species, binding to three direct 9-bp repeats of the consensus sequence A-C-T/G-T/G-T/G-T/C-A-A/G-C was reported, with spacer regions of 5 bp and 35 bp (8). In contrast to the *phoRS* operon of *C. glutamicum*, the *phoPR* operon of *M. tuberculosis* is negatively autoregulated, and binding of PhoP to the *phoP* promoter appears to be independent of phosphorylation (8).

With respect to the transcriptional start site, the PhoR-binding site is centered at position -185 in the *pstSCAB* promoter and at position -52 in the *phoRS* promoter. Whereas the latter position allows a direct interaction of PhoR with the α subunit of RNA polymerase or with the sigma factor, DNA bending or an interaction with additional regulatory proteins would be required for such an interaction in the *pstSCAB* promoter. As mentioned above, evidence for another PhoR-binding site in the *pstSCAB* promoter located between -99 and -136 was obtained (Fig. 5). A search in this region for the occurrence of the optimal binding motif (Fig. 7, no. 3) revealed the sequence TTTGTGATTAGAGTCTCCA (positions -135 to -117) as the best hit (Fig. 7, no. 4), but the match was weak. Transcriptional fusion studies showed that the DNA region between -194 and -176 upstream of the transcriptional start site is essential for PhoR-dependent induction of the *pstSCAB* operon in vivo, and therefore, the region from -135 to -117 alone is not sufficient (25). The in vivo relevance of the PhoR-binding site in the *phoRS* promoter was also supported by transcriptional fusion studies, as shown in Fig. 9.

A search for the occurrence of the currently best 19-bp PhoR-binding site in the upstream regions of the other proposed PhoR target genes *phoC*, *ushA*, *porB*, *ugpAEBC*, *pitA*, *nucH*, *phoH*, and *glpQ1* revealed that all contained one or two sequences with similarity to the query motif (Fig. 7, no. 4 to 15). Whether they indeed represent PhoR-binding sites has yet to be confirmed. Besides a definition of the PhoR regulon, an understanding of the physiological function of the PhoRS two-component system requires knowledge of the stimulus sensed by the histidine kinase PhoS, a topic to be addressed in future studies.

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