The SphS–SphR two-component system is the exclusive sensor for the induction of gene expression in response to phosphate limitation in *Synechocystis*

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**Running title:** Responses to phosphate limitation in *Synechocystis*
SUMMARY

Living organisms respond to phosphate limitation by expressing various genes whose products maintain an appropriate range of phosphate concentrations within each cell. We identified previously a two-component system, which consists of histidine kinase SphS and its cognate response regulator SphR, that regulates the expression of the phoA gene for alkaline phosphatase under phosphate-limiting conditions in the cyanobacterium Synechocystis sp. PCC 6803. In the present study, we used DNA microarrays to investigate the role of SphS and SphR in the regulation of the genome-wide expression of genes in response to phosphate limitation. In wild-type cells, phosphate limitation strongly induced the expression of 12 genes with induction factors greater than 7. These genes were included in three clusters of genes, namely, the pst1 and pst2 clusters that encode phosphate transporters; the phoA gene and the nucH gene for the extracellular nuclease. Phosphate limitation strongly repressed the expression of only the urtA gene with induction factors below 0.2. Inactivation of either of SphS or SphR completely eliminated the phosphate limitation-inducible expression of the 12 genes and the phosphate limitation-repressible expression of the urtA gene. These results suggest that the SphS-SphR two-component system in Synechocystis sp. PCC 6803 is the dominant sensory system that controls gene expression in response to phosphate limitation.
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INTRODUCTION

Phosphate is an essential nutrient and it is required, for example, for the biosynthesis of nucleotides, ATP, DNA and RNA, and for the functional regulation of proteins by phosphorylation. However, phosphate is one of the least available nutrients in the environment. Therefore, regulatory mechanisms have developed for the acquisition, storage, and metabolism of phosphate. One such regulatory mechanisms involves the induction of the expression of genes for proteins that participate in the uptake of phosphate and/or increase the availability of phosphate under phosphate-limiting conditions. The induced expression of such genes has been observed under phosphate-limiting conditions in eubacteria, such as *Escherichia coli* (1) and *Bacillus subtilis* (2); in cyanobacteria, such as *Synechocystis* sp. PCC 6803 (hereafter, *Synechocytis*; 3) and *Synechococcus* sp. PCC 7942 (hereafter, *Synechococcus*; 4); in yeast (5); and in plants (6, 7). The primary event under such conditions is the perception of phosphate-limiting conditions and transduction of the signal. This system has been studied in prokaryotes and appears to be a two-component signal-transduction system, consisting of a histidine kinase and a response regulator.

In *E. coli*, a histidine residue of a specific histidine kinase, PhoR, is phosphorylated in response to phosphate limitation. The phosphate group is then transferred to a specific aspartate residue on the cognate response regulator.
PhoB, which regulates the expression of genes in the Pho regulon that encode alkaline phosphatase, phosphate transporters, and other relevant proteins (1). In *B. subtilis*, the expression of genes in the Pho regulon is regulated by a two-component signal-transduction system that consists of a histidine kinase, PhoR, and a response regulator, PhoP (2).

In a previous study, we identified two genes in *Synechocystis*, namely, *s110337* (*sphS; hik7*) and *s1r0081* (*sphR; rre29*), that encode proteins that are homologous, respectively, to PhoR and PhoB of *E. coli*, to PhoR and PhoP of *B. subtilis*, and to SphS and SphR of *Synechococcus* (3). We found that the SphS-SphR two-component system induced the expression of alkaline phosphatase in response to phosphate limitation. However, we did not determine whether this two-component system regulates only some or all aspects of phosphate limitation-inducible gene expression in *Synechocystis*.

In the present study, we used DNA microarrays to analyze the genome-wide expression of genes in *Synechocystis* that occurs in response to phosphate limitation. We found that the expression of 12 genes was strongly induced while that of only one gene was strongly repressed by phosphate limitation. We also found that the expression of all the phosphate limitation-inducible genes was completely eliminated upon inactivation of either SphS or SphR. In addition, we found that SphR bound to the upstream flanking regions of three genes at repetitive *PyTTAAPyPy(T/A)*-like sequences. Our observations suggest that
the SphS-SphR two-component system in *Synechocystis* might be the only system for the perception and transduction of the phosphate-limitation signal in *Synechocystis*.
EXPERIMENTAL PROCEDURES

Strains and mutants — Synechocystis sp. PCC 6803 was provided by Dr. J.G.K. Williams (Dupont de Nemours Co., Inc., Wilmington, DE). The \[sphs] mutant was constructed by insertional mutagenesis of the \[sphs] gene using a spectinomycin-resistance gene cassette, as described previously (8; the terminology is that recommended on the CyanoBase website http://www.kazusa.or.jp/cyanobase/Synechocystis/mutants/cgi-bin/comshow.cgi?id=s110337).

The \[sphr] mutant was generated by insertional mutagenesis of the \[sphr] gene using a kanamycin-resistance gene cassette, as follows. The complete \[sphr] gene was amplified by PCR using genomic DNA extracted from wild-type cells of Synechocystis with ISOLANT II (Nippon Gene, Tokyo, Japan) as the template, the forward primer 5′-GGATCCATGTTGAAATACCATCT-3′ and the reverse primer 5′-CTCGAGCTAAACGATAGCCCA-3′. The 801-bp product of PCR was cloned into pT7Blue (Novagen, Madison, WI), to generate pT7sphr. This plasmid was sequenced with an automated DNA sequencer (ABI310; Perkin-Elmer Biosystems, Foster City, CA) to confirm the sequence of the product of PCR. A kanamycin-resistance gene cassette was transposed into \[sphr] using the Tn5 transposition system of an EZ::TN\textsuperscript{TM} <KAN-2> Insertion Kit (Epicentre Technologies, Madison, WI) according to the manufacturer’s instruction. The site at which the kanamycin-resistance gene cassette had been inserted was

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determined by sequencing the regions flanking the kanamycin-resistance gene cassette and it was found to be 384 bp downstream of the site of the initiation of translation. The resultant plasmid was designated pT7sphR::Km<sup>R</sup> and used for transformation of *Synechocystis* as described previously (9). To examine the extent of replacement of the *sphS* and the *sphR* genes by the corresponding mutated gene in mutant cells, we amplified the genes using the chromosomal DNA from wild-type, ∆*sphS*, and ∆*sphR* cells and pairs of synthetic primers for the *sphS* and *sphR* genes, respectively.

*Culture conditions*— Wild-type and mutant cells were grown at 34 °C in BG-11 medium (10) as described previously (11). Phosphate-limiting conditions were established by centrifugation and gel filtration as follows. A culture of *Synechocystis* cells in BG-11 medium was centrifuged at 25 °C at 3,000 x g for 5 min. The pellet was resuspended in 3 ml of phosphate-free BG-11 medium, in which K$_2$HPO$_4$ (0.18 mM) had been replaced by an equimolar solution of KCl (0.18 mM) to give a concentration of Cl<sup>-</sup> ions of 0.31 mM. To remove phosphate completely, the suspension was passed through a desalting column (PD-10; Amersham Biosciences, Piscataway, NJ; bed volume, 8.3 ml; radius 0.73 cm; height 5.0 cm) that had been filled with Sephadex G-25 and had been equilibrated with phosphate-free BG-11 medium. The flow-through fraction that contained *Synechocystis* cells was collected and inoculated into phosphate-free BG-11 medium. Then cells were cultured as
described above.

**DNA microarray analysis** — An aliquot of culture was withdrawn rapidly and cells were killed immediately by addition of an equal volume of ice-cold ethanol that contained 10% (w/v) phenol. Total RNA was extracted as described previously (12) and then cDNAs, labeled with fluorescent dyes (Cy3-dUTP and Cy5-dUTP; Amersham Pharmacia Biotech), were prepared from 5 µg of total RNA with an RNA fluorescence labeling core kit (*Moloney murine leukemia virus* version 2.0; TAKARA Biomedicals, Kyoto, Japan). DNA microarrays (CyanoCHIP Version 1.6) were purchased from TAKARA Biomedicals. After hybridization, each microarray was rinsed and scanned as described previously (12, 13).

**Overproduction of His-tagged SphR in *E. coli and its purification** — We digested plasmid pT7sphR with BamHI and XhoI and cloned the resultant fragment into the plasmid vector pET28a (Novagen) for subsequent expression of a fusion protein with a His tag at the amino terminus. The product was designated pETsphR and used for the transformation of *E. coli* BL21(DE3)pLysS. Transformed cells were grown at 37 °C in 500 ml of LB medium (1.0% tryptone, 0.5% yeast extract, and 171 mM NaCl) supplemented with 50 µg/ml kanamycin. When the absorbance at 600 nm of the culture reached 0.8, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the culture was incubated for an additional 12 h at 20 °C. The cells were collected by centrifugation at 4 °C at 10,000 x g for 10 min and the pelleted cells were stored at -80 °C prior
to use.

All procedures for purification of the fusion protein were performed at 4 °C. The transformed and pelleted *E. coli* cells were suspended in 40 ml of 50 mM Tris-HCl buffer (pH 8.0) that contained 0.3 M NaCl and then they were disrupted with a French press (SLM Instruments, Inc., Rochester, NY) at 8.3 MPa. The homogenate was centrifuged at 10,000 x g for 20 min. The resultant supernatant was loaded onto a column (Poly-Prep Chromatography column; Bio-Rad Laboratories, Hercules, CA; bed volume, 1 ml) that had been filled with Ni-NTA agarose (QIAGEN, Hilden, Germany) and had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0) that contained 0.3 M NaCl. The matrix was washed with 30 ml of 50 mM Tris-HCl buffer (pH 8.0) that contained 0.3 M NaCl and 50 mM imidazole and then with 10 ml of 50 mM Tris-HCl buffer (pH 8.0) that contained 50 mM NaCl and 50 mM imidazole. Finally, the fusion protein was eluted, in 0.5 ml fractions, in 50 mM Tris-HCl buffer (pH 8.0) that contained 50 mM NaCl and 250 mM imidazole. Fractions that contained the fusion protein were combined and desalted by passage through a desalting column (PD-10; Amersham Biosciences), which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0) that contained 5 mM EDTA (disodium salt), 50 mM KCl, and 10% (v/v) glycerol. The eluate was frozen in liquid N$_2$ and stored at -80 °C prior to use. The concentration of proteins was determined with a BD protein assay kit (Bio-Rad), with bovine serum albumin as the standard. The purity of the fusion protein was examined by
SDS-PAGE (14).

*Gel mobility shift assay* — A 294-bp DNA fragment that corresponded to positions -294 to -1 upstream of the site of the initiation of translation of the *phoA* gene of *Synechocystis* was amplified by PCR with the genomic DNA from wild-type cells of *Synechocystis* as template, the forward primer 5'-TTTTAAGCTTTCCGCGAGGATTTTTCGC-3' and the reverse primer 5'-TTTTAAGCTTAATTGCTTATGAAATTTCCTC-3'. Each primer contained a HindIII restriction site. The resultant fragment of DNA was digested with HindIII and was then end-labeled with [32P]-dCTP (6,000 Ci/mmol; Amersham Biosciences) by the Klenow fragment of DNA polymerase (TAKARA Biochemicals). Unincorporated radioisotope was removed by gel-filtration chromatography on a Centri-Sep spin column (Perkin-Elmer Biosystems, Foster City, CA). Approximately 10 ng (0.05 pmol) of the end-labeled DNA fragment (~2,600 cpm) were mixed with 0.5 μg (30 pmol) of His-tagged SphR, which had previously been incubated in the presence of 10 mM acetyl phosphate or in its absence in 20 μl of 50 mM Tris-HCl buffer (pH 8.0) that contained 1 mM EDTA, 150 mM KCl, 1 mM DTT, 5 μg/ml poly(dI-dC) (Amersham Biosciences), and various nonlabeled DNA fragments, such as the region upstream of the site of initiation of translation of the *phoA*, *sphX*, *pstS2*, and *mtnC* genes, and a DNA fragments of 30 bp that had been synthesized by reference to the sequence of the Pho box of the *sphX* gene of *Synechocystis* and a similar but base-substituted fragment (see Results). DNA fragments of 302,
500, and 135 bp, corresponding to positions -299 to +3, -500 to -1, and -135 to -1 upstream of the site of the initiation of translation of the *sphX*, *pstS2*, and *mntC* genes, respectively, were synthesized by PCR with the genomic DNA from wild-type cells of *Synechocystis* as template, and following primers: the forward primer 5′-AAAAAAGCTTGGTAAATCGGGCAAAAAGTC-3′ and reverse primer 5′-AAAAAAGCTTCAATTTTCATCCTTCGCC-3′ for *sphX*; forward primer 5′-TTTTAAGCTTGGCAGTCGTGATG-3′ and reverse primer 5′-GGGGAAGCTTGGCAGTGCAATCTAAATTG-3′ for *pstS2*; and forward primer 5′-

GGGGCTCGAGCAAGTATTAGTGTCAG-3′ and reverse primer 5′-

TTTTAAGCTTAGTGATAATGGCTTTCAT-3′ for *mntC*. The DNA fragments of 30 bp that corresponded to the Pho box and the base-substituted Pho box were synthesized by annealing the synthetic oligonucleotides 5′-TTTAACCAACCTTTACTAGGGCTTAACCT-3′ and 5′-TCCGTTGAAACTCCCTGAGGGCCCGTGT-3′, respectively, to their complementary oligonucleotides. Each reaction mixture for the gel mobility shift assay was incubated at 25 °C for 30 min and then loaded onto a 6% polyacrylamide gel. Electrophoresis was performed at 4 °C in TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Then the gel was dried and analyzed with a Bio-imaging analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan).

*Complementation of \( sphS \) and \( sphR \) mutants* — The *sphS* gene was amplified with the genomic DNA from wild-type cells of *Synechocystis* as template, the forward primer 5′-

GTCGACGATCTACTGATGCGCGATTC-3′ and the reverse primer 5′-
CCCAGGGGCACTTAAAAATATCGCTC-3’. These sequences were located 277 bp upstream of the initiation codon and 167 bp downstream of the termination codon, respectively. The forward and reverse primers contained restriction sites for SalI and SmaI, respectively. The sphR gene was amplified similarly with the forward primer 5’-GTCGACGGGCAAAAACCTAAACCGTCC-3’ and the reverse primer 5’-CTCAGCTCCAGATAAGTAAATATGCG-3’. These sequences were located 182 bp upstream of the initiation codon and 100 bp downstream of the termination codon, respectively. The primers contained restriction sites for SalI and XhoI, respectively. The resultant fragments were cloned into the TA cloning site of plasmid pT7Blue, as noted above, yielding plasmids pT7sphSC and pT7sphRC, which were sequenced with the automated DNA sequencer to confirm the sequences of the products of PCR.

For the construction of complementation vectors, we excised sphS and sphR genes that included the flanking regions from pT7sphSC and pT7sphRC with SalI plus SmaI and SalI plus XhoI, respectively. Each fragment was inserted into the cyanobacterial autonomous replication plasmid pVZ321 (15), which had been digested with the corresponding restriction enzymes. The resultant complementation vectors, namely, pVZ321::sphSC and pVZ321::sphRC, were introduced into ΔsphS and ΔsphR cells, respectively, by triparental gene transfer (15).
RESULTS

Genome-wide analysis with DNA microarrays of gene expression in response to phosphate limitation — Preliminary control experiments with DNA microarrays, using Cy3- and Cy5-labelled cDNAs derived from a single sample of RNA extracted from wild-type cells grown in normal medium, indicated that the limit of experimental deviation corresponded to as much as a twofold difference in relative levels of expression (12, 16). Therefore, genes demonstrating more than twofold induction or repression were defined as phosphate limitation-inducible or -repressible genes, respectively, in subsequent analyses.

Genes whose expression, over time, was induced or repressed more than seven-fold in wild-type cells under phosphate-limiting conditions are listed in Table I (20 min, 1 h, 2 h, 4 h, and 8 h). Phosphate limitation strongly induced the expression of the Pho regulon, which includes genes for two types of phosphate transporter, alkaline phosphatase, and an extracellular nuclease. There are two sets of genes for putative phosphate-specific transport (Pst) systems in the genome of Synechocystis (17). Both Pst systems, designated Pst1 and Pst2, are encoded by similar clusters of genes (Fig. 1A). The Pst1 system includes six ORFs (sll0679-sll0684) in the following order: sphX-pstS1-pstC1-pstA1-pstB1-pstB1'. The Pst2 system includes four ORFs (slr1247-slr1250) as follows: pstS2-pstC2-pstA2-pstB2. The phoA gene for alkaline phosphatase and the nucH gene for the extracellular nuclease, both of which are
involved in increasing the availability of phosphate in the extracellular environment, are located in tandem on the genome of *Synechocystis* (Fig. 1A).

Phosphate limitation also repressed the expression of a number of genes. The expression of the *urtA* gene for a periplasmic protein of unknown function was strongly repressed in response to phosphate limitation. This gene is located at a different site from the phosphate limitation-inducible genes on the genome of *Synechocystis* (Fig. 1B).

*Rapid and slow responses* — As noted above, both *Pst* systems were induced by phosphate limitation in *Synechocystis*. However, induction of the expression of the six genes in *Pst1* occurred within 20 min of exposure to phosphate-limited conditions and the levels of expression of these genes remained high for 8 h (Table I). By contrast, the responses of the four genes in *Pst2* occurred much more slowly. Induction became apparent at 1 h and transcripts reached a maximum level at 8 h. The expression of the *phoA* gene and the *nucH* gene occurred only slowly (Table I) and the phosphate limitation-repressible gene, *urtA*, responded slowly too. A total of 8 h was required before the level fell to a minimum value.

*Effects of inactivation of SphS or SphR on the phosphate limitation-regulated expression of genes* — We previously identified histidine kinase SphS and the corresponding response regulator SphR as a two-component system that regulates the expression of the *phoA* gene for alkaline phosphatase under
phosphate-limiting conditions in *Synechocystis* (3).

Fig. 2A shows the positions at which the spectinomycin-resistance gene cassette and the kanamycin-resistance gene cassette were inserted in the *sphS* and *sphR* genes by targeted mutagenesis. *Synechocystis* contains approximately ten copies of the chromosome per cell (18). To determine whether the *sphS* and *sphR* genes had been disrupted in every copy, we extracted the genomic DNA from the mutant cells and used it as template for the amplification of each gene by PCR. Fig. 2B shows that no copies of the parental genes were present in the mutant cells, suggesting that the replacement of *sphS* and *sphR* genes by mutated genes was complete in each respective mutant.

We examined first the effects of these mutations on gene expression when cells were grown with sufficient phosphate, namely, in normal BG-11 medium. We observed that the expression of the *cysS* gene, which encodes cysteinylation-tRNA synthetase, was repressed twofold upon inactivation of either SphS or SphR. The expression of *slr0915*, which encodes an endonuclease, and the expression of the *pstS1* gene were enhanced and repressed, respectively, twofold upon the inactivation of SphR. Although the expression of these genes was affected by mutations in SphS and/or SphR, the changes in levels of expression were only minor or close to the extent of experimental deviations.

Table II shows the effects of phosphate limitation during incubation for 8 h on gene expression in wild-type, [sphS], and [sphR] cells. Inactivation of SphS or SphR completely eliminated
the phosphate limitation-regulated expression of genes both at 20 min and at 8 h, indicating that both the rapid and the slow responses to phosphate limitation were regulated by the SphS-SphR two-component system.

**Binding of SphR to the upstream flanking region of phosphate limitation-inducible genes via recognition of specific sequences** — The response regulators PhoB in *E. coli* and PhoP in *B. subtilis* bind specifically to conserved sequences, known as Pho boxes (19, 20). Although the conserved sequences and spacings of the Pho boxes are different in *E. coli* and *B. subtilis*, the general structure is conserved in these microorganisms. The Pho box of *E. coli* consists of 7-bp direct repeats of CTGTCAT-like sequences that are separated by 4 bp (19), whereas the Pho box of *B. subtilis* consists of 6-bp direct repeats of TTAACA-like sequences that are separated by 5 bp (20).

In order to investigate the possibility that *Synechocystis* might also have a Pho box, we examined the sequences of the upstream flanking regions of the phosphate limitation-inducible genes. Although we did not find a sequence that was identical to those of the Pho boxes of *E. coli* and *B. subtilis*, we did find repetitive 8-bp sequences, namely, PyTTAAPPyPy(T/A), that corresponded to a consensus sequence in the upstream flanking regions of the *phoA*, *sphX*, and *pstS2* genes. In *Synechocystis*, these sequences were separated by 3 bp (Fig. 3A).

To determine whether SphR can bind to the upstream flanking regions of the various genes, we overexpressed SphR in *E. coli*
as a soluble fusion protein with a His tag at its amino terminus. The relative molecular mass of the His-tagged SphR (His-SphR), after purification by affinity chromatography, was estimated by SDS-PAGE to be 33.4 kDa, which was the same as that deduced from the amino acid sequence of the fusion protein.

We examined the DNA-binding activity of His-SphR in gel mobility shift assays with a $^{32}$P-labeled fragment of DNA that corresponded to the flanking region from positions $-294$ to $-1$ upstream of the codon for the initiation of translation of the $phoA$ gene (Fig. 3A). We detected a band that was due to formation of a complex between His-SphR and the upstream flanking region of the $phoA$ gene (Fig. 3B, lane 2, arrow c). In the presence of a 10-fold or 100-fold excess of the nonlabeled fragment of the DNA that corresponded to the upstream flanking regions of the $phoA$, $sphX$, and $pstS2$ genes, the extent of the binding of His-SphR to the labeled fragment of DNA was reduced significantly (Fig. 3B, lanes 3, 4, 5, 6, 7, and 8). By contrast, the presence of a 10-fold or 100-fold excess of a nonlabeled fragment of DNA that corresponded to the upstream flanking region of the $mntC$ gene, which encodes a subunit of the ABC-type manganese transporter and is not a phosphate limitation-responsible gene, did not affect the binding of His-SphR to the labeled DNA (Fig. 3B, lanes 9 and 10). These results suggest that SphR was able to bind specifically to the upstream flanking regions of the $phoA$, $sphX$, and $pstS2$ genes.

In order to confirm that the consensus sequence
PyTTAAPyPy(T/A) corresponded to the Pho box of *Synechocystis*, we examined the ability of His-SphR to bind to a 30-bp synthetic fragment of DNA that contained three such repeats and to a 30-bp fragment that contained three repeats in which the conserved sequence had been replaced by a different sequence. Fig. 4A shows the sequences of the two 30-bp fragments. In the base-substituted fragment, PyTTAAPyPy(T/A) was changed to PyCCGCTG(T/A). In gel motility shift assays, we detected a band that was due to the binding of His-SphR to the upstream flanking region of the *phoA* gene (Fig. 4B, lane 2, arrow c). In the presence of a 10-fold or 100-fold excess of the nonlabeled “wild-type” fragment of 30 bp, the extent of the binding of His-SphR to the labeled DNA was reduced (Fig. 4B, lanes 3 and 4). By contrast, the presence of a 10-fold or 100-fold excess of the nonlabeled base-substituted 30-bp fragment did not affect the binding of His-SphR to the labeled DNA (Fig. 4B, lanes 5 and 6). These results indicated that SphR was able to bind specifically to PyTTAAPyPy(T/A) repeats. We examined the effects of incubation of SphR with 10 mM acetyl phosphate, conditions for phosphorylation of the Asp residue in SphR, on its DNA-binding activity. However, this treatment did not affect the DNA-binding activity of SphR (data not shown).

Complementation of □SphS and □SphR mutants — In order to confirm that the SphS-SphR two-component system serves as the only system for sensing phosphate limitation, we introduced wild-type *sphS* and *sphR* genes into □*sphS* and □*sphR* mutant cells
using pVZ321::sphSC and pVZ321::sphRC, respectively. DNA microarray analysis confirmed that the expression of the wild-type genes in the respective mutant cells restored the capacity for phosphate limitation-inducible expression of genes in response to phosphate-limiting conditions (data not shown). These results confirmed that the effects on the phosphate limitation-induced expression of genes shown in Table II were caused by the mutations in the sphS and sphR genes.
DISCUSSION

Rapid and slow responses to phosphate limitation in Synechocystis — DNA microarray analysis revealed that the expression of 12 genes was strongly enhanced under phosphate-limiting conditions (Table I). These genes encode proteins that are involved in phosphate transport and increases in phosphate availability (Table I).

In B. subtilis, both the phosphorylated and the dephosphorylated forms of PhoP are able to bind to promoters of the Pho regulon but only the phosphorylated form stimulates the initiation of transcription (21). Moreover, the initiation of transcription requires a certain level of phosphorylated PhoP, and the level is specific to each individual promoter. For example, the initiation of transcription of the phoA gene requires a three-fold higher level of phosphorylated PhoP than does that of the pstSCAB operon (22). The rapid response of the sphX promoter and the slower responses of other promoters in Synechocystis to phosphate limitation might be explained in a similar way, namely, by requirements for different levels of phosphorylated SphR for the initiation of transcription. It is possible that the level of phosphorylated SphR 20 min after the transfer of cells to phosphate-limited conditions was low but sufficient for the initiation of transcription of the Pst1 genes, even though it was insufficient for the initiation of transcription of the Pst2 genes and of the other phosphate limitation-inducible genes in Synechocystis.
The region upstream of the site of initiation of translation, which might include the promoters of phoA, sphX, and pstS2, contained repeats of the PyTTAAPPyPy(T/A) consensus sequence (Fig. 3A). Gel mobility shift assays indicated that SphR was able to bind to DNA fragments that corresponded to the putative promoter regions of the phoA, sphX, and pstS genes (Fig. 3B). These fragments contained direct repeats of PyTTAAPPyPy(T/A) that were separated by 3 bp (Fig. 3A). Moreover, SphR was also able to bind to a synthetic DNA fragment of 30 bp that contained three such repeats (Fig. 4), a result that suggests that this repetitive PyTTAAPPyPy(T/A) sequence might be a putative Pho box in *Synechocystis*. In *Synechococcus*, the binding sites of the promoters of the phoA and sphX genes to the response regulator SphR do, in fact, contain four repeats similar to the putative Pho box of *Synechocystis* (4). In addition, we found repetitive PyTTAAPPyPy(T/A)-like sequences in the upstream flanking regions of gene clusters that correspond to the Pst systems in *Anabaena* sp. PCC 7120 and *Thermosynechococcus elongatus* BP-1. While we found four such repeats in the upstream flanking regions of the phoA gene and the pstS2 gene, there are only three repeats in the upstream flanking region of the sphX gene (Fig. 3A). Our DNA microarray analysis demonstrated that the extent of induction of the expression of both the phoA and pst2 genes was much higher than that of pst1 genes (Table I). Four repeats of TTAACA-like sequences are required for efficient binding of PhoP to the pstS promoter in *B. subtilis* (23). Therefore, it
is likely that the extent of induction of phosphate limitation-inducible genes in *Synechocystis* depends on the number of repeated PyTTAAPyPy(T/A) sequences.

*Phosphate limitation-repressible gene in Synechocystis* — DNA microarray analysis revealed that phosphate limitation strongly repressed the expression of the *urtA* gene (Table I). This repression was regulated by the SphS-SphR two-component system in *Synechocystis* (Table II). In *B. subtilis*, the *tagAB*, *tagDEF*, and *resDE* operons are repressed by the PhoPR two-component system in response to phosphate limitation (2). The expression of these operons is repressed by the phosphorylated form of PhoP, which binds to regions that overlap the sites of initiation of transcription and extend further into the coding regions of these genes. However, we found no PyTTAAPyPy(T/A)-like sequences in these regions of the *urtA* gene. Furthermore, SphR did not bind to DNA fragments that corresponded to the upstream flanking region and coding region of the *urtA* gene. Further studies are necessary to identify the molecular mechanism by which SphR represses the expression of the *urtA* gene under phosphate-limiting conditions.

*A hypothetical pathway for the perception and transduction of phosphate-limitation signals in Synechocystis* — Computational analysis predicts that SphS has neither a hydrophobic domain nor a periplasm-targeting signal sequence, suggesting that it is a soluble protein located in the cytoplasm. This protein has a strongly conserved histidine kinase domain
in its carboxy-terminal region and a PAS domain near its amino
terminus. The PAS domain is involved in the sensing of a variety
of stimuli, such as oxygen (24), light (25), and redox potential
(26). It has been suggested that the PAS domain might be involved
in protein-protein interactions that mediate signal
transduction and in the determination of the specificity of such
interactions (27). SphR belongs to the OmpR family of response
regulators. It consists of a strongly conserved receiver domain
in the amino-terminal region and a DNA-binding domain, with a
helix-turn-helix motif, in the carboxy-terminal region.

Fig. 5 shows a hypothetical scheme for the perception and
transduction of phosphate-limitation signals. In this
hypothetical scheme, when the amino-terminal input module of
SphS senses an intracellular decrease in the level of phosphate,
the conserved histidine residue in the histidine kinase domain
is phosphorylated. The phosphate group is then transferred to
the aspartate residue in the receiver domain of SphR. As the
result of this phosphorylation, SphR is activated and is able
to regulate the expression of genes.

A two-component system homologous to SphS-SphR is
conserved in six of the eight cyanobacterial species whose
genomes have been sequenced, namely, *Synechocystis*, *Anabaena*
sp. PCC 7120, *T. elongatus* BP-1, *Gloeobacter violaceus* PCC 7421,
*Prochlorococcus marinus* MED4, and *Synechococcus* sp. WH 8102.
It has not been found in *P. marinus* SS120 and *P. marinus* MIT9312.
A Blast search of all non-redundant protein database
(http://www.ncbi.nlm.nih.gov/BLAST/) indicated that this system is conserved in approximately 80 of 125 bacterial strains whose genomes have been sequenced. These findings indicate that this two-component system is rather widely distributed in the prokaryotic kingdom.

The molecular mechanism by which SphS perceives the phosphate-limitation signal is still unclear. Further studies are necessary to identify the sensing domain of SphS and to characterize the phosphorous moiety or moieties that are sensed by the sensory kinase.
ACKNOWLEDGEMENTS

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REFERENCES


LEGENDS TO FIGURES

FIG. 1. **Genes and gene clusters whose expression was regulated by phosphate limitation in *Synechocystis*.** Arrows indicate directions of transcription and lengths of open reading frames. Shaded arrows correspond to genes whose expression was strongly regulated (induction factor ≥ 7) by phosphate limitation. Open arrows correspond to genes whose expression was unregulated or only marginally regulated (induction factor < 7) by phosphate limitation. (A) Gene clusters with inducible expression. (B) A gene with repressible expression.

FIG. 2. **Insertional mutagenesis of the sphS and sphR genes in *Synechocystis*.** (A) A schematic representation of insertional mutagenesis with the spectinomycin-resistance (*Sp*<sup>r</sup>) and kanamycin-resistance (*Km*<sup>r</sup>) gene cassettes. The numbers indicate the positions of base pairs, counted from the site of initiation of translation. Small arrows indicate the positions of the primers for analysis by PCR of native and mutated sphS and sphR genes. (B) Determination by PCR of the extent of replacement of wild-type (WT) genes by mutated genes, demonstrating that the replacement was complete in both cases. Lane M, size markers.
FIG. 3. Nucleotide sequences of upstream flanking regions of phoA, sphX, and pstS2 genes and results of gel mobility shift assay of the binding of SphR to the upstream region of phoA gene. (A) Sequences of upstream flanking regions of phoA, sphX, and pstS2 genes, showing putative SphR-binding sites. Numbers indicate the positions of base pairs, counted from the putative site of initiation of translation. Consensus sequences are shown in boldface letters. (B) Gel mobility shift assay of the binding of His-SphR to the upstream flanking region of phoA gene and competition by DNA fragments that corresponded to the upstream flanking regions of the sphX, pstS2, and mntC genes. His-SphR and a DNA fragment of 294 bp that corresponded to positions −294 to −1 upstream of the site of initiation of translation of the phoA gene were synthesized and the fragment was labeled with $^{32}$P as described in Experimental Procedures. The labeled fragment of DNA (10 ng) was incubated in a volume of 20 μl with 0.5 μg of His-SphR and with nonlabeled DNA fragments of 294, 500, 302, and 135 bp that corresponded to positions −294 to −1, −500 to −1, −299 to +3, and −135 to −1 upstream of the site of initiation of translation of phoA, sphX, pstS2, and mntC genes, respectively. Lane 1, no His-SphR; lane 2, no nonlabeled DNA; lane 3 and 4, 100 ng and 1 mg, respectively, of a nonlabeled DNA fragment of phoA; lane 5 and 6, 100 ng and 1 mg, respectively, of a nonlabeled DNA fragment of sphX; lane 7 and 8, 166 ng and 1.66 mg, respectively, of a nonlabeled fragment of pstS2; lane 9 and 10, 47 ng and 470 ng, respectively, of a nonlabeled fragment of
mntC. Arrows c and f indicate the position of the His-SphR-bound and free fragments, respectively, of the upstream flanking region of phoA gene.

**FIG. 4. Synthetic DNA fragments of the Pho box and the base-substituted Pho box of Synechocystis and results of the gel mobility shift assay of the binding of SphR to the upstream flanking region of the phoA gene.** (A) Sequences of the synthetic DNA fragments of 30 bp that represented the Pho box of the sphX gene of Synechocystis and the corresponding base-substituted Pho box. Synthetic Pho box corresponded to the putative SphR-binding site of the sphX gene. Numbers indicate the positions of base pairs, counted from the putative site of initiation of translation of the sphX gene. Consensus sequences are shown in boldface letters. The base-substituted nucleotides are underlined. (B) Gel mobility shift assay of the binding of SphR to the upstream flanking region of the phoA gene and competition by the 30-bp synthetic Pho box and the base-substituted Pho box. Experiments were performed as described in the legend to Fig. 3. Lane 1, no His-SphR; lane 2, no nonlabeled DNA; lane 3 and 4, 10 ng and 100 ng, respectively, of the nonlabeled synthetic Pho box; lane 5 and 6, 10 ng and 100 ng, respectively, of the nonlabeled base-substituted Pho box. Arrows c and f indicate the position of the His-SphR-bound and free fragments, respectively, of the upstream flanking region of the phoA gene.
FIG. 5. A hypothetical scheme for the perception and transduction of phosphate-limitation signals in *Synechocystis*. The histidine kinase domain, the PAS domain, the receiver domain, and the DNA-binding domain are indicated by a gray rectangle, a rectangle with tiny squares, a hatched rectangle, and an open rectangle, respectively. Histidine and aspartate residues that might be involved in the phosphorelay reaction are indicated by H and D in circles, respectively. Genes that are enclosed within a specific reactangle form a cluster on the *Synechocystis* genome (see Fig. 1).
TABLE I

Identification by DNA microarray analysis of genes whose expression is affected by phosphate limitation in wild-type Synechocystis

Wild-type cells of Synechocystis were depleted of phosphate as described in Experimental Procedures and then incubated in the absence of phosphate for 8 h under normal growth conditions. At the indicated times, an aliquot of the suspension of cells was withdrawn and cells were killed immediately and subjected to DNA microarray analysis as described in Experimental Procedures. Each value indicates the ratio of the level of mRNA from cells that had been grown under phosphate-limiting conditions to that from cells that had been grown in normal BG-11 (phosphate-sufficient conditions). Genes that gave ratios greater than 7-fold are listed. The numbering of open reading frames (ORF) and the annotation of genes correspond to those in the Cyanobase (http://www.kazusa.or.jp/cyano/). The data presented here are averages of results from two independent experiments, each of which included a duplicate set of DNA microarrays.
<table>
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<tr>
<th>ORF</th>
<th>Gene</th>
<th>Product</th>
<th>Phosphate-limited BG-11/BG-11 (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 min</td>
</tr>
<tr>
<td>sll0679</td>
<td><em>sphX</em></td>
<td>ABC-type phosphate transporter phosphate-binding protein</td>
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<table>
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<th>Genes showing more than 5-fold repression (-fold)</th>
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<tr>
<td>slr0447 <em>urtA</em> Periplasmic protein</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
TABLE II

DNA microarray analysis of the effects of a mutation in SphS or SphR on the phosphate limitation-regulated expression of genes in Synechocystis

Experiments were performed as described in Table I. Each value is the ratio of the level of a specific mRNA from cells that had been grown under phosphate-limiting conditions for 20 min or 8 h, as indicated, to that from cells that had been grown in normal BG-11 (phosphate-sufficient conditions). Only genes that gave ratios greater than 7-fold in the analysis of wild-type cells are included in the Table. The data presented here are averages of results of two independent experiments.
<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
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<th>ΔsphR</th>
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- 36 -
A

\[ \text{pstB1' pstA1 pstS1} \]
\[ \text{pstB1 pstC1 sphX} \]
\[ \text{pst1} \]

\[ \text{pst2} \]
\[ \text{pstC2 pstB2} \]
\[ \text{pstS2 pstA2} \]

\[ \text{nucH phoA} \]

B

\[ \text{urtA} \]
Suzuki et al. Fig. 2

A

\[ \text{slr0370} \quad \text{sphS} \quad (\text{sll0337}) \quad \text{slr0336} \]

\[ \text{slr0080} \quad \text{sphR} \quad (\text{slr0081}) \quad \text{slr0082} \]

B

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<th>Primers for sphS</th>
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<tr>
<td>M</td>
<td>WT</td>
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</tbody>
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- 4 kb
- 3 kb
- 2 kb
- 1 kb
A

**phoA**

-175  
CTTAACCTTTTACATGCTAAACCAAGTTTTCTTAAATTT

-135  

**sphX**

-146  
CTCTCCAGCCCCCTTAACCAAACCTTTATAGGGCTTTAACC

-106  

**pstS2**

-342  
CTTAATCTTTCTTAATTCCTATCTTAATTTCCGACTTTAATCA

-303  

B

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<td>2</td>
<td>3</td>
<td>4</td>
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</tbody>
</table>

- 39 -
A

Pho box (sphX)

\[-135 \quad -106\]

TTTAACCAACCTTTACTAGGGCTTAACCT

Base-substituted Pho box

TCCGGTGAAACCCGGTGAGGGCCCCTG

B

<table>
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\[\text{c} \quad \text{f}\]
Phosphate limitation

SphS

SphR

PAS

H

DNA binding

Rapid

sphX
pstS1
pstC1
pstA1
pstB1
pstB1'

Slow

phoA
nucH

pstS2
pstC2
pstA2
pstB2

Slow

urtA
The SphS-SphR two-component system is the exclusive sensor for the induction of
gene expression in response to phosphate limitation in Synechocystis
Shingo Suzuki, Ali Ferjani, Iwane Suzuki and Norio Murata

J. Biol. Chem. published online January 5, 2004

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