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2 Function of the N-terminal region of the phosphate-sensing
3 histidine kinase, SphS, in *Synechocystis* sp. PCC 6803

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10 Running title: Involvement of a PAS domain for Pi-sensing by SphS

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12 The contents category: Cell and Molecular Biology of Microbes

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17 Footnote

18 Abbreviations: AP: alkaline phosphatase, PAS: Per-Arnt-Sim, TM: transmembrane

Summary

In *Synechocystis* sp. PCC 6803 the histidine kinase SphS (*sll0337*) is involved in transcriptional activation of the phosphate (Pi)-acquisition system which includes alkaline phosphatase (AP). The N-terminal region of SphS contains both a hydrophobic region and a Per-Arnt-Sim (PAS) domain. The C-terminal region has a highly conserved transmitter domain. Immunological localization studies on heterologously expressed SphS in *Escherichia coli* indicate that the hydrophobic region is important for membrane localization. In order to evaluate the function of the N-terminal region of SphS, deletion mutants under the control of the native promoter were analyzed for *in vivo* AP activity. Deletion of the N-terminal hydrophobic region resulted in loss of AP activity under both Pi-deficient and Pi-sufficient conditions. Substitution of the hydrophobic region of SphS with that from Ni²⁺-sensing histidine kinase, NrsS, resulted in the same induction characteristics as SphS. Deletion of the PAS domain resulted in the constitutive induction of AP activity regardless of Pi-availability. To characterize the PAS domain in more in detail, four amino acid residues conserved in the PAS domain were substituted with Ala. Among the mutants R121A constitutively expressed AP activity, suggesting that R121 is important for the function of the PAS domain. Our observations indicated that the presence of a transmembrane helix in the N-terminal region of SphS is critical for activity and the PAS domain is involved in perception of Pi-availability.

INTRODUCTION

Phosphorus is an essential element for living organisms and is present in cells predominantly as phosphate. Inorganic phosphate (Pi) is incorporated into many cellular components including nucleotides, nucleic acids, phospholipids and a large variety of metabolic intermediates. It is also important in the covalent modification of proteins via phosphorylation which is an essential component of many signal transduction and regulatory pathways. Despite its importance phosphorus is one of the least available nutrients in the environment (Schweitzer & Simon, 1995; Tyrrell, 1999). The responses brought about by Pi-deficiency in *Escherichia coli* have been extensively studied. A two-component signal transduction system is required for regulation of the expression of the *pho*-regulon (Makino *et al.*, 1989), which encodes proteins for acquisition, storage, and utilization of Pi.

Two-component signal transduction systems consist of two proteins, a histidine kinase and a response regulator (Stock *et al.*, 1989). Typically histidine kinases are membrane-bound sensory proteins. Once a specific stimulus is perceived by the kinase, a conserved histidine residue in the protein is autophosphorylated and the phosphate group transferred to a cognate response regulator. The activity of the response regulator is determined by phosphorylation. Since many response regulators affect transcription, this provides a way in which the two-component system can modulate physiological responses to intra- and extra-cellular changes. The regulatory pathway is further complicated by the discovery of a third component modulating the activity of specific two-component systems (Chen *et al.*, 2008; Salinas *et al.*, 2007). The Pi-responsive two-component system is present in a wide variety of bacteria. In *E. coli* it consists of the histidine kinase PhoR, and the response regulator PhoB (Makino *et al.*, 1989). Disruption of *phoB* results in complete loss of response to Pi-deficiency, consistent with

the two-component system being essential for induction of the *pho*-regulon. A third component, PhoU, is also involved in regulation of the *pho*-regulon. Inactivation of *phoU* constitutively activates expression of the *pho*-regulon via PhoR-PhoB regardless of Pi-availability (Nakata *et al.*, 1984). In *E. coli*, PhoU thus functions as a negative regulator of the two-component system under Pi-sufficient conditions. Under Pi-deficient conditions the inhibitory effect of PhoU is overcome and the PhoR-PhoB sensory relay activity is restored, resulting in expression of the *pho*-regulon.

In general, histidine kinase proteins consist of two portions of a signal-input domain located at the N-terminus and a transmitter domain located at the C-terminus (Mascher *et al.*, 2006). The former perceives the specific stimulus and modulates the activities of autophosphorylation and phospho-transfer to the cognate response regulator located in the latter domain. Analysis of the N-terminal region in sensory histidine kinases is important in order to understand the mechanism of signal perception. In *E. coli*, the N-terminal region of PhoR possesses two transmembrane (TM) helices and a Per-Arnt-Sim (PAS) domain (Scholten *et al.*, 1993). Deletion of the TM region and/or the PAS domain from PhoR results in constitutive activation of the transmitter domain irrespective of Pi-availability. This suggests that both domains are involved in repression of kinase activity under Pi-sufficient conditions (Scholten *et al.*, 1993; Yamada *et al.*, 1990). In *Bacillus subtilis* the PhoR protein also contains two TM helices and a PAS domain. However it has a large periplasmic loop between the TM helices which is absent from PhoR in *E. coli*. In *B. subtilis* deletion of the two TM helices and the periplasmic loop from the PhoR protein does not affect induction of the *pho*-regulon suggesting that the cytosolic transmitter domain senses Pi-availability and induces expression of the *pho*-regulon (Shi & Hulett, 1999). The molecular mechanism for sensing the availability of Pi and regulating it via a histidine kinase thus clearly differs

between organisms and the role of specific protein domains warrants further investigation.

Histidine kinases and response regulators are expressed at a very low level and this is likely to be an important factor in downstream gene regulation. Over-accumulation of histidine kinases and response regulators in cells can cause excess signal transduction between cognate partners or between non-cognate partners. (Ehira & Ohmori, 2006; Krall & Reed, 2000). This phenomenon has been utilized to screen for novel histidine kinase activities. For example over-expression of the histidine kinase SasA from the cyanobacterium *Synechococcus* sp. PCC 7942, which functions as a regulator of circadian rhythm, functionally complements the EnvZ and PhoR mutations in *E. coli* (Nagaya *et al.*, 1993) which function as osmosensors and phosphosensors respectively.

Investigation into the specific function of histidine kinases and the role of component domains should thus be conducted under conditions of gene expression equivalent to those of the native histidine kinases in wild-type cells in order to prevent erroneous conclusions. Previous analyses of the signal input domains of PhoR in *E. coli* and in *B. subtilis* have utilized multicopy plasmids with strong promoters to express the kinases and it would be advisable to avoid this.

In order to express genetically modified kinases at equivalent levels to the native protein *in vivo*, the coding sequence of the native gene in the chromosome could be replaced with genes encoding the target proteins via homologous recombination and driven from the original promoter. Since the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) readily undergoes double homologous recombination between the transformed DNA fragment and the chromosomal DNA (Williams, 1988), we decided to exploit this ability to study the function of sub-domains of the Pi sensing histidine kinases in this organism utilizing the original promoter. Additionally since

most of the genes for histidine kinases in *Synechocystis* are transcribed in a monocistronic manner there is no need to take care of the expression levels of the genes downstream from the histidine kinase as they are not cotranscribed (Murata & Suzuki, 2006). These characteristics of *Synechocystis* greatly simplify studies of genetically modified histidine kinases.

The genome of *Synechocystis* encodes the histidine kinase SphS (Slr0337) and the response regulator SphR (Slr0081) and a negative regulator SphU (Slr0741), which are structural and functional orthologs of PhoR, PhoB and PhoU respectively (Aiba *et al.*, 1993; Hirani *et al.*, 2001; Juntarajumnong *et al.*, 2007a; Suzuki *et al.*, 2004). In *Synechocystis* these genes are located on the genome separately and should be transcribed independently. The SphS-SphR pathway regulates the expression of at least 12 genes, including the *phoA* gene which encodes a periplasmic alkaline phosphatase (AP) (Suzuki *et al.*, 2004). Induction of AP activity is a highly conserved response to Pi-deficiency in many bacteria and has been used as an indicator of the activity of the SphS-SphR pathway in *Synechocystis* (Juntarajumnong *et al.*, 2007a). Burut-Archanai *et al.* (2009) have demonstrated that deletion of a portion of the N-terminal sequence of SphS results in constitutive induction of AP activity, indicating that this region is essential to respond to Pi fluctuations.

In this report we describe an expression system in *Synechocystis* cells for modified forms of SphS utilizing the native promoter of the *sphS* gene so eliminating any artifacts due to over-production. We have expressed a series of SphS derivatives and assessed the signal sensing ability of the modified SphS by measuring cellular AP activity. The possible functions of the hydrophobic region and the PAS-like domain of SphS are discussed.

METHODS

Bacterial strains and culture conditions. A glucose-tolerant strain of *Synechocystis* (Williams, 1988) was used as the wild-type strain. The wild-type strain and its derivatives were grown at 34°C in BG11 medium (Stanier *et al.*, 1971) buffered with 20 mM HEPES-NaOH (pH 7.5) under continuous illumination provided by incandescent lamps at 70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Phosphate-free BG11 medium was prepared by substitution of K_2HPO_4 with KCl as described previously (Suzuki *et al.*, 2004). Mutants of SphS were grown in the presence of 25 μg kanamycin ml^{-1} or 25 μg spectinomycin ml^{-1} , depending on the inserted antibiotic resistance gene. Liquid cultures were continuously bubbled with 1.0% (v/v) CO_2 -containing air. *E. coli* strains JM109 (TaKaRa Bio), and BL21(DE3) (Merck KGaA), were used as hosts for genetic manipulation and protein expression respectively, and were grown in LB medium with appropriate antibiotics at 37°C (Miller, 1972).

Genetic modifications of the SphS gene in *Synechocystis*. To replace the entire ORF of the *sphS* gene with a kanamycin-resistance cassette, DNA fragments upstream and downstream of the ORF of *sphS* were amplified using PCR with upstream primers, 0337uF and 0337uR, and downstream primers, 0337dF and 0337dR, using genomic DNA of *Synechocystis* as a template. Primer sequences used in this study were shown in Supplementary Table S1. The upstream fragment was cloned between the *NsiI* and *NdeI* site of pGEM[®]-T easy vector (Promega). This was followed by cloning of the downstream fragment between the *SalI* and *SacII* sites of the plasmid containing the upstream fragment to obtain pSK01. All plasmids constructed in this study and their descriptions were shown in Table 1. A kanamycin-resistance cassette, containing *NdeI* and *SalI* sites at each end, was amplified by PCR using kanF and kanR primers and

EZ-Tn5 (Epicentre) as template. The amplified DNA fragment was digested with *NdeI* and *SalI* and inserted into pSK01, which had been cleaved with the same enzymes, to construct pSK02. To obtain the *sphS*-deleted strain, wild-type cells of *Synechocystis* were transformed with pSK02 as described previously (Williams, 1988).

In order to replace the kanamycin-resistance cassette with modified *sphS* genes, a DNA fragment containing the sequence of transmitter domain of SphS was amplified by PCR using kinF1 and 0337R primers and cloned between the *HindIII* and *SalI* sites of pSK02 to obtain pSK03. A spectinomycin-resistance gene cassette, containing *SalI* sites at both ends, was amplified by PCR using speF and speR primers and the *aadA* gene in pAM1146 (Tsinoremas *et al.*, 1994) as template. This fragment was cloned into the *SalI* site of pSK03 to obtain pSK04.

The full-length *sphS* gene was amplified by PCR using 0337F and 0337R primers to construct the complementary strain (SphSC). The SphS construct lacking a hydrophobic-region at the N-terminus (*sphSΔHy*) was obtained by PCR using primers ΔhyF and 0337R. To construct the PAS domain-deleted SphS (*sphSΔPAS*), the partial *sphS* sequences were amplified by PCR using primer pairs 0337F plus PASuR, and PASdF plus 0337R. The two fragments were mixed and further amplified by PCR using 0337F and 0337R primers generating a 0.95 kbp DNA fragment. The PCR products were cloned into pGEM[®]-T easy vector and the DNA sequences were confirmed. Each of the DNA fragments cloned into pGEM[®]-T easy vector were excised from the vector with *NdeI* and *HindIII* and cloned between *NdeI* and *HindIII* sites of pSK04 to obtain pSK05, pSK06, and pSK07 for SphSC, SphSΔHy and SphSΔPAS strain, respectively (Fig. S1).

To construct strain SphSΔN, expressing the signal-input domain deleted SphS, the DNA fragment corresponding to the transmitter domain of SphS was amplified by PCR using

kinF2 and 0337R primers. The fragment was cloned between the *Nde*I and *Sal*I sites of pSK01, and the spectinomycin-resistance cassette was inserted into the plasmid as described above to construct pSK08. Δ SphS cells were transformed with the plasmids constructed above and spectinomycin-resistant cells were selected.

Construction of fusion-protein expressing strain. To express a fusion protein of the N-terminal part of the nickel-sensing histidine kinase from *Synechocystis*, NrsS (Lopez-Maury *et al.*, 2002), and SphS, a DNA fragment corresponding to the N-terminal region (amino acid residues 1 to 222) of *nrsS* was amplified by PCR using primer pair nrsSF and nrsSR. The resultant fragment was cloned into the *Nde*I site of pSK06 or pSK08 to construct pSK09 and pSK10 (Fig. S2). The direction of the inserted fragment was confirmed by PCR using nrsSF and 0337R primers. Δ SphS cells were transformed with pSK09 or pSK10 to yield the NrsS(N)-SphS Δ Hy and NrsS(N)-SphS Δ N strains respectively.

Construction of strains expressing point-mutated SphS. In order to substitute the conserved amino acid residues in the PAS domain of SphS with Ala, the *sphS* containing the targeted substitution was synthesized by a fusion PCR strategy (Wang *et al.*, 2002). This resulted in the point-mutated *sphS* fragments (Fig. S3). The fragments were then digested with *Nde*I and *Xho*I and cloned between the *Nde*I and *Xho*I sites of pSK04. Δ SphS cells were transformed with the plasmids, and the strains, SphS(P80A), SphS(N96A), SphS(R121A), SphS(E124A), and SphS(H207A) were obtained.

Measurement of AP activity. Wild-type and mutant strains of *Synechocystis* grown in BG11 medium for 16 h were harvested by centrifugation at 25°C at 3,000 x g for 5 min

and the cells were washed twice with phosphate-free BG11 medium. The cells were subsequently inoculated into BG11 or phosphate-free BG11 medium at an OD 730 nm of 0.2 to 0.4 and further incubated for 24 h. The activity of AP in the intact cells was measured by the rate of degradation of *p*-nitrophenyl phosphate (Aiba *et al.*, 1993).

Preparation of recombinant SphS protein and raising an antibody. The coding region of the *sphS* gene in pSK05 was cleaved by *Nde*I and *Sal*I and cloned into pET28a (Merck KGaA) digested with both enzymes. The plasmid, pET-SphS, was then introduced into *E. coli* BL21(DE3) cells (Fig. S3).

E. coli transformants were grown at 37°C in LB medium supplemented with 25 µg kanamycin ml⁻¹. The recombinant protein was induced in exponentially grown cells by 1 mM IPTG. After 4 h of incubation cells were harvested by centrifugation, washed with Buffer A [50 mM HEPES-NaOH (pH 7.5), 100 mM NaCl], and suspended in Buffer A. The cells were then disrupted by sonication at 4°C using a UD-201 sonicator (TOMY). The cell extract was centrifuged at 20,000 x *g* for 10 min and the inclusion body containing recombinant protein was collected. This was washed twice with Buffer B [50 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 5 mM EDTA, 0.05% (w/v) sodium deoxycholate, 0.5 mg lysozyme ml⁻¹] at room temperature for 30 min. The inclusion body was suspended in Buffer C [50 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 8 M Urea]. Insoluble material was removed by centrifugation and the supernatants containing His-tagged SphS was loaded onto a HiTrap chelating column (GE Healthcare Bio-Science) and washed with Buffer C. Bound proteins were eluted with Buffer C containing 250 mM imidazole.

Eluted proteins were subjected to electrophoresis on 12.5% SDS-PAGE and the gel was stained as described previously (Ortiz *et al.*, 1992). A band, corresponding to the

recombinant protein, was excised and the gel slice crushed and suspended in electrophoresis buffer (Laemmli, 1970). Protein was eluted from the gel by shaking for 12 h at room temperature, filtered with a 5 μ m centrifugal filter device (Ultrafree-MC, Millipore) and the purified recombinant SphS was used to immunize a rabbit to obtain an antibody against SphS.

Localization of SphS in *E. coli*. To express SphS, SphS Δ Hy and NrsS(N)-SphS Δ Hy in *E. coli*, we constructed pSK16, 17, and 18 which includes the entire sequence for SphS or modified SphS substituting the phosphorylatable His residue with Ala under control of the strong artificial *trc* promoter (Fig. S4). Mutation of the His residues in SphS derivatives was included to inactivate the kinase activity so minimizing possible effects of over-production of the kinases on the growth of *E. coli* cells. To exchange the H207 of SphS Δ Hy with alanine, the DNA fragment between *Xba*I and *Xho*I of pSK06 was exchanged with that of pSK15 and the resulting plasmid pSK06a was produced. In order to introduce the *trc* promoter into pSK15 and pSK06a, a DNA fragment including the *trc* promoter was amplified by PCR using *trcF* and *trcR* primers and pTrc99A (Amann & Brosius, 1985) as template. After digesting the amplified DNA fragment with *Nde*I, it was cloned into the *Nde*I site of pSK15 and pSK06a and the resulting plasmids were named pSK16 and pSK17, respectively. The direction of the inserted *trc* promoter was confirmed by PCR. To construct the plasmid pSK18 the *trc* promoter sequence was amplified by PCR using *trcF2* and *trcR* primers and pTrc99a DNA as the template. The amplified DNA was digested with *Nsi*I and *Nde*I, and inserted between the *Nsi*I and *Nde*I sites of pSK17. The amplified *nrsS* DNA fragment as described above was digested with *Nde*I and introduced into the *Nde*I site of the resulting plasmid. Plasmids pSK16, pSK17, pSK18, and a control plasmid pSK05 were introduced into *E.*

coli strain JM109 and the transformants were cultured in LB medium at 37°C. Cells in exponential growth phase were collected by centrifugation, resuspended in two different buffers systems [a] Buffer D [50 mM Tris-HCl (pH 8.0), 50 mM NaCl] or [b] Buffer E [Buffer D containing 100 mM Na₂CO₃ (pH 11.5)] at 4 °C and disrupted by sonication. Clear cell lysates obtained after removal of cell debris by centrifugation at 20,000 x g for 10 min were then ultra-centrifuged at 160,000 x g, for 1 h at 4 °C. The supernatant was withdrawn and termed the cytosolic protein fraction. The precipitates were washed by suspending in the same buffer used for sonication and recovering by ultra-centrifugation under the same conditions. In order to distinguish between membrane bound proteins and insoluble matters which were not removed by the initial centrifugation, the final pellet was resuspended in Buffer F [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1% (v/v) Tween20] and removed insoluble matter by centrifugation. The detergent-soluble fraction was designated the membrane proteins.

For Western blot analysis, proteins separated by SDS-PAGE were electro-transferred to PVDF membrane. Rabbit anti-SphS serum and alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad) were used as the primary and secondary antibodies. The primary antibody was used at a 1:1,000 dilution with TBS (Tris-Buffered Saline, 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl) containing 1% (w/v) skim milk and the secondary antibody was used at a 1:20,000 with TBS. Detection was carried out using the amplified Alkaline Phosphatase GAR kit according to manufacturer's instruction (Bio-Rad). 10 µg of protein from each fraction were subjected to 10% SDS-PAGE. Protein concentrations were determined by Protein Assay Kit (Bio-Rad) with bovine serum albumin as standard.

RESULT AND DISCUSSION

Construction of domain-deleted SphS expressing strains. Juntarajumnong *et al.* (2007b) have reported that the translation start site of the *sphS* gene is located at the ATG 141 bp upstream from the GTG originally assigned as the translation initiation codon (Kaneko *et al.*, 1996, <http://bacteria.kazusa.or.jp/cyanobase/>). The newly annotated SphS consists of 430 amino acid residues and contains a hydrophobic region at the N-terminal region, a transmitter domain at the C-terminal region, and a putative PAS domain between them (Fig. 1). This is a structure which many orthologs of SphS have. To investigate molecular mechanisms of signal perception by SphS, we decided to express the domain-deleted SphS from the original promoter of the *sphS* gene and examine its responsiveness *in vivo* using the AP reporter protein activity. This strategy ensures that the level of the sensor molecule is maintained at the same level as the wild type. To express genetically modified SphS in cells of *Synechocystis* from the native promoter of the *sphS* gene, the entire ORF of the *sphS* was substituted with a kanamycin-resistance gene cassette (Fig. 1a). Segregation of the *sphS*-deleted chromosome was confirmed by PCR (data not shown) and the strain, named Δ SphS, was utilized as a host for secondary transformations introducing modified SphS. The kanamycin-resistance gene of Δ SphS was replaced with the modified *sphS* genes containing a spectinomycin-resistance gene cassette which was inserted immediately downstream of the termination codon of *sphS*. (Fig. S1). The strains SphS Δ N, SphS Δ Hy and SphS Δ PAS contained deletions in the entire N-terminal region (amino acid residues 2-196) excluding the transmitter domain, the hydrophobic region (amino acid residues 2-50), and the putative PAS domain (amino acid residues 69-177) respectively. The intact *sphS* gene was also introduced to obtain a complementary strain SphSC. Replacement of all the Δ SphS chromosomal copies with the inserted genes was

confirmed by PCR and sensitivity to kanamycin (data not shown). All the spectinomycin-resistant strains grew well in Pi-sufficient conditions (data not shown).

Effects of the domain-deletions of SphS on the expression of AP activity. Since the expression of the gene for AP is solely regulated by the SphS/SphR pathway in *Synechocystis* (Suzuki *et al.*, 2004), we assayed AP activity to evaluate functionality of the SphS derivatives (Fig. 1b). The AP activity in cells of Δ SphS was negligible under both Pi-sufficient and -deficient conditions. AP activity in response to Pi-availability in the SphSC strain was similar to that observed in the wild type. This indicates that SphSC was functioning normally regardless of base substitutions introduced in the upstream and the downstream coding regions introduced to insert the gene constructs into *NdeI* and *SalI* sites, these substitutions did not alter the amino acid sequence. Similarly, insertion of a spectinomycin-resistance gene cassette downstream of the coding region did not cause any alterations to the normal function of the gene. We next analyzed the AP activities in response to Pi-availability in cells of the domain-deleted strains. SphS Δ N and SphS Δ Hy did not exhibit any AP activity at any level of Pi-availability. This suggests that the hydrophobic domain in the N-terminal region of SphS is essential for sensing Pi-availability and/or activating kinase activity *in vivo*. Cells of SphS Δ PAS constitutively expressed AP activity regardless of Pi-availability, as has been reported in cells of the *sphU*-deleted strain (Juntarajumnong *et al.*, 2007a), suggesting that the PAS domain might function to suppress SphS activity under Pi-sufficient conditions, possibly together with SphU. The construct containing the hydrophobic domain minus the PAS domain [SphS Δ PAS] exhibited constitutive kinase activity. However the construct minus the hydrophobic domain and containing the PAS domain [SphS Δ Hy] was inactive. This indicates that the hydrophobic domain is

essential for activation of the transmitter domain.

Signal perception/ kinase activation only requires a hydrophobic region and is not

sequence specific. To investigate the dependence of the specific amino acid sequence of the SphS hydrophobic domain on kinase activation and sensing we decided to completely replace this region with the amino acid sequence from the Ni-sensing histidine kinase, NrsS. These two hydrophobic regions have very little amino acid sequence similarity (Fig. S5). We constructed strains that expressed a fusion protein with the N-terminal region of a Ni²⁺-sensing histidine kinase, NrsS (Lopez-Maury *et al.*, 2002), and the truncated forms of SphS to obtain NrsS(N)-SphS Δ N and NrsS(N)-SphS Δ Hy (Fig. 2a). NrsS up-regulates the *nrsBACD* operon coding for an ABC transporter involved in Ni²⁺-tolerance in the presence of Ni²⁺ in *Synechocystis*. NrsS has two membrane-spanning helices at the N-terminal region and is predicted to be located in the plasma membrane (Lopez-Maury *et al.*, 2002). If the hydrophobic domain of SphS is essential in sensing changes in the Pi-availability, replacement with the hydrophobic region from NrsS may result in loss of Pi-sensing.

AP activity in the cells of NrsS(N)-SphS Δ Hy responded to Pi-availability to the same extent as SphSC. However, whilst SphSC did not have any AP activity under Pi-sufficient conditions, detectable activity was present in NrsS(N)-SphS Δ Hy. We also looked at AP activity in the cells of NrsS(N)-SphS Δ N, which has the PAS domain deleted, and like SphS Δ PAS, the activity was constitutive (Figs. 1 and 2).

These findings suggest that the hydrophobic region of SphS might not be essential for signal perception and/or kinase activation. It also indicates that the presence of any hydrophobic region may be sufficient for induction of AP activity by SphS.

The N-terminal region of SphS is essential for membrane localization. To investigate the region of SphS that may be required for membrane localization, and hence probably signal perception, we designed a series of constructs which were truncated/deleted in specific domains. We also generated an antibody that was capable of recognizing all of the domains in the protein. Following expression and fractionation into membrane and soluble fractions it should be possible to determine the location of the protein immunologically.

The antibody could specifically detect the recombinant SphS via Western blotting at a level of 5 ng (data not shown). At first, we attempted to detect SphS protein in wild-type cells. Cells in exponential growth phase were collected and disrupted by sonication. After removing the unbroken cells and cell debris, the cell lysate was separated into cytoplasmic, plasma membrane, and thylakoid membrane fractions by discontinuous sucrose density gradient centrifugation (Murata and Omata, 1988). After 10 μ g of each sample was subjected to Western blotting analysis, no protein was detected. This indicates that the amount of SphS *in vivo* is below the detection limit (data not shown) and is perhaps not surprising as sensor proteins are liable to be of very low abundance.

We next attempted to over-express SphS in *Synechocystis*. The Δ SphS strain was transformed with plasmids pSK16 or pSK17, which contain the *sphS* and *sphS Δ Hy* gene, respectively, under the control of *P_{trc}*, a strong bacterial artificial promoter (Fig. S4). To regulate expression levels of the introduced gene, the *lacI^q* gene was concomitantly integrated into the chromosomes via homologous recombination (Fig. S4). To minimize unexpected effects of the over-expression of the sensor proteins, the phosphorylatable His residues of both proteins were substituted with an Ala residue. After transformation, spectinomycin-resistant colonies of both transformants were obtained. However, we could not cultivate the cells in liquid BG11 medium containing 25 μ g spectinomycin

ml⁻¹ even without addition of IPTG. The increased level of SphS in these cells may be causing deleterious effects on cellular function. The precise mechanism for this is unclear. Since elevated expression of SphS, and its derivatives, in *Synechocystis* was deleterious we decided to investigate expression in a heterologous system. SphS, SphSΔHy and NrsS(N)-SphSΔHy were successfully expressed in *E. coli* cells and fractionated for immunological determination of subcellular localization. We used two different pH treatments for preparation of the soluble and membrane fractions, pH 8.0 and pH 11.5. The latter pH was employed to overcome any problems which might be associated with proteins being trapped inside membrane vesicles. Treatment of membranes at pH 11.5 is a well documented method of linearizing closed membrane vesicles (Griff *et al.*, 1992). If a positive result was obtained on pH 8.0 isolated membranes and a negative one with pH 11.5 membranes this would indicate that the result was not due to membrane insertion but rather trapping of the protein inside a closed vesicle. True membrane insertion could only be verified if both the protein was detected in pH 8.0 and pH 11.5 preparations. All the membrane preparations isolated at pH 8.0 gave a positive signal (Fig. 3). SphSΔHy could be solubilized after the pH 11.5 treatment, indicating that it is not membrane inserted. SphS and NrsS(N)-SphSΔHy, which contain the hydrophobic domain, remained membrane bound after pH 11.5 treatment consistent with the hydrophobic domain being essential for membrane localization.

Identification of critical amino acid residues in the PAS domain. Our results above indicate that the PAS domain might be involved in the regulation of kinase activity dependent on the Pi-availability (Figs. 1, 2, and 4). To identify amino acid residues required for PAS domain function, we aligned PAS domains from several orthologs of

413 SphS, including PhoR from *E. coli* (Fig. 4a) and identified 4 absolutely conserved
 414 hydrophilic residues which could be critically important. It is noteworthy that the PAS
 415 domains of cyanobacterial SphS proteins have lower identity with that of PhoR, but the
 416 function is still conserved. Thus the conserved amino acid residues might have an
 417 important role for the function of the domain. The four conserved residues P80, N96,
 418 R121 and E124, and autophosphorylatable His residues, H207, in SphS, were separately
 419 substituted with Ala and introduced into the Δ SphS *Synechocystis* cells. Point mutations
 420 of P80A and E124A did not influence the induction of AP activity, suggesting that these
 421 residues are not critical for activity of the PAS domain in SphS (Fig. 4b). Mutations of
 422 N96A and H207A resulted in loss of induction of AP activity regardless of
 423 Pi-availability. N96 might be important for the activation of the transmitter domain
 424 since substitution resulted in complete inactivation. Mutation of R121A caused
 425 constitutive expression of the AP activity even under Pi-sufficient conditions,
 426 suggesting that R121 might have an important function in sensing by the PAS domain.
 427 In *E. coli*, PhoR lacking its TM region constitutively expresses the AP gene, suggesting
 428 that the region is required for negative regulation of PhoR activity. In SphS of
 429 *Synechocystis*, deletion of part of the hydrophobic region (8-15 amino acid residues)
 430 also constitutively induces AP expression (Burut-Archanai *et al.*, 2009). Our results
 431 indicate that deletion of the entire hydrophobic region of SphS was unable to activate
 432 reporter AP activity regardless of Pi-availability. Moreover, replacement of the
 433 hydrophobic region from NrsS into SphS retained Pi-responsiveness, suggesting that the
 434 hydrophobic region might not play a role in sensing the Pi-signal but rather may be
 435 important in localizing the sensory protein to a specific location on the membrane.
 436 There are several examples where histidine kinases in bacteria are located at the poles of
 437 cells (Hallez *et al.*, 2007). The hydrophobic region of SphS might be important to

localize it to a specific location. In order to detect SphS expressed *in vivo* in *Synechocystis* new techniques will have to be developed.

In *E. coli*, it is suggested that the activity of PhoR is repressed through formation of a hetero complex with PhoU, and the high-affinity Pi transporter (Wanner, 1993). No direct evidence of such protein interaction has been demonstrated (Baek *et al.*, 2007). In this study we have found that recombinant SphS expressed in *E. coli* was localized to the membrane via the hydrophobic domain. This suggested that a similar situation might occur in *Synechocystis* but we were not able to demonstrate it because of the low amount of SphS in *Synechocystis*. The presence of this domain is required for activation of kinase activity. The repressive function of the PAS domain under Pi-sufficient conditions was similar to that of the SphU protein. It may be that the SphU protein interacts with SphS via the PAS domain. This could be tested by looking for direct protein-protein interactions. The mutants which we have generated affecting the function of the PAS domain, as R121A and N98A, could be very useful materials to demonstrate the interaction between SphS and SphU genetically.

ACKNOWLEDGMENTS

We are grateful to Prof. A.R. Slabas and Dr. J. Rowland (University of Durham, UK) for their constructive discussion. This work was partially supported by Grant-in-Aid for Scientific Research on Priority Areas (17051032).

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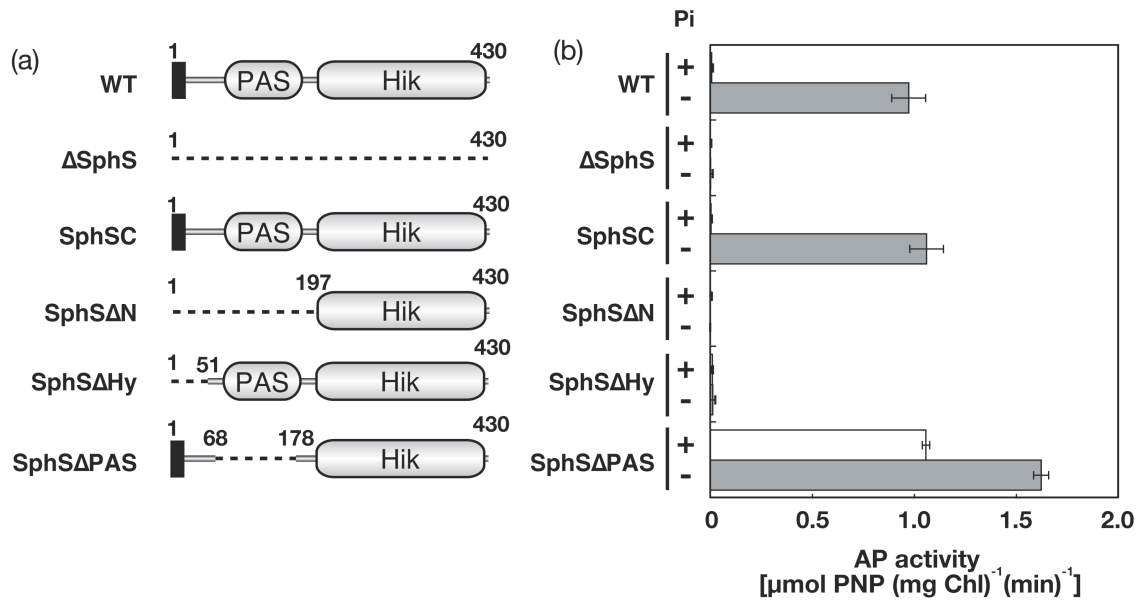


Fig. 1. Effects of deletion of domains in SphS on the expression of AP activity. Domain architectures of native and mutated SphS (a). The domains were depicted with Pfam program (Fin *et al.*, 2008). Numbers indicate the positions of amino acid residues from the translation start site of the native SphS. Dotted lines represent the deleted regions from the native protein. PAS, Hik and black rectangle indicate PAS domain, transmitter domain of histidine kinase and the hydrophobic region, respectively. AP activities in each strain grown under Pi-sufficient (+Pi, open bar) or Pi-deficient (-Pi, shaded bar) conditions for 24 h (b). The assays were carried out three times with cultures grown independently.

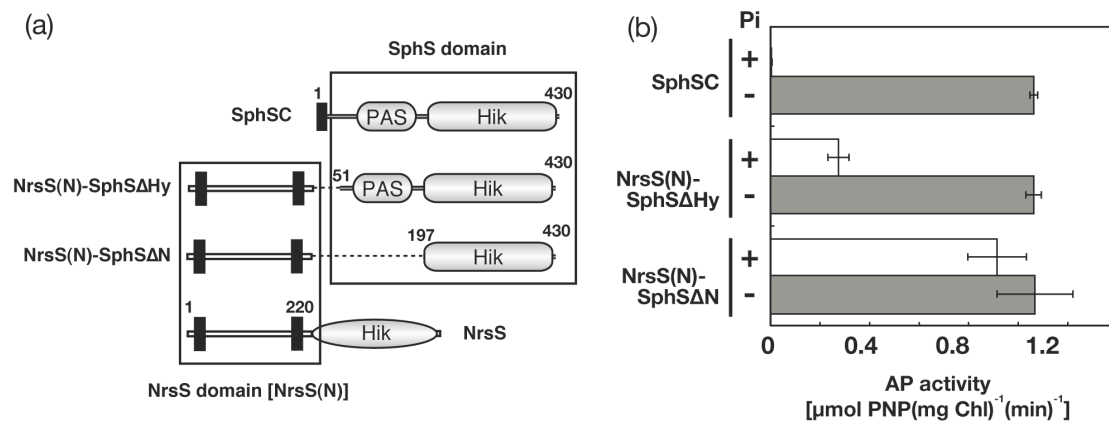


Fig. 2. Fusions of truncated SphS constructs with the TM region of NrsS. Domain architectures of the fusion proteins (a) and AP activities under Pi-sufficient (+Pi, open bar) or Pi-deficient (-Pi, shaded box) conditions (b). The assays were carried out three times with cultures grown independently.

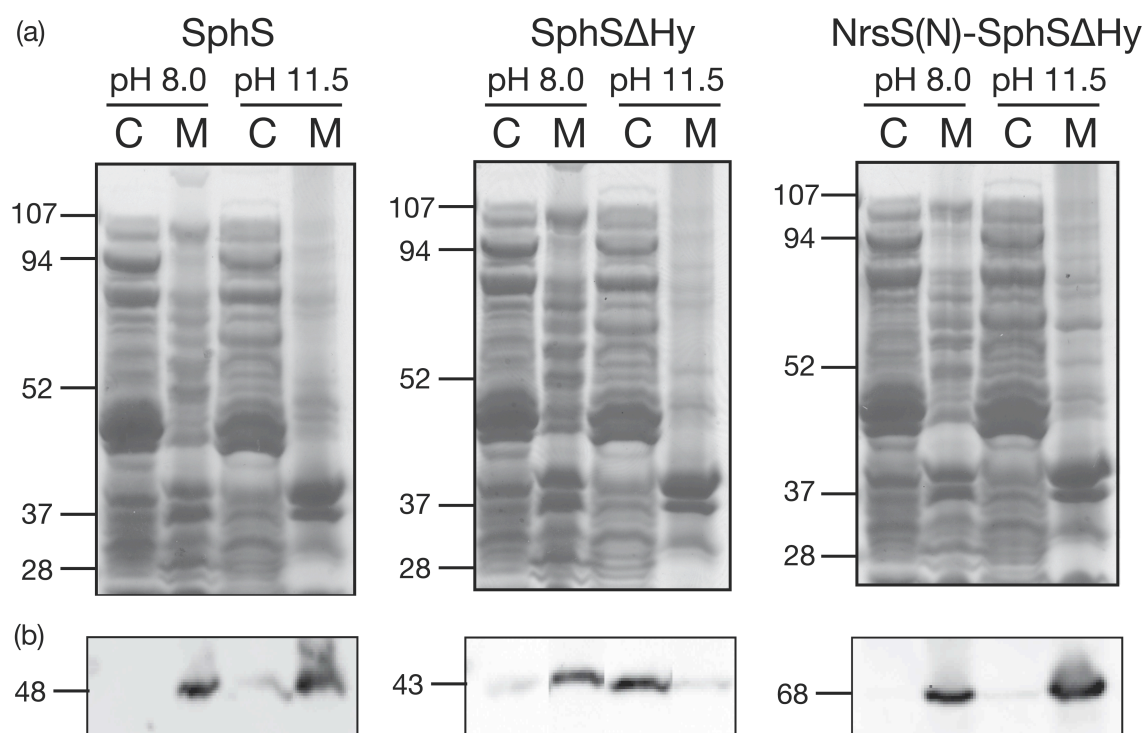


Fig. 3. Western blot analysis of SphS and modified SphS proteins expressed in cells of *E. coli*. Cells of *E. coli* expressing the native and modified SphS proteins were disrupted at pH 8.0 and at pH 11.5 conditions. The crude extracts were further separated by ultracentrifugation into cytosolic (C) and membrane (M) fractions. 10 μ g of each fraction was subjected to SDS-PAGE. Gels were stained with CBB (a) or electroblotted on PVDF membranes and detected with an anti-SphS polyclonal antibody (b). These results were reproducible and a representative result was presented. Numbers indicate molecular mass.

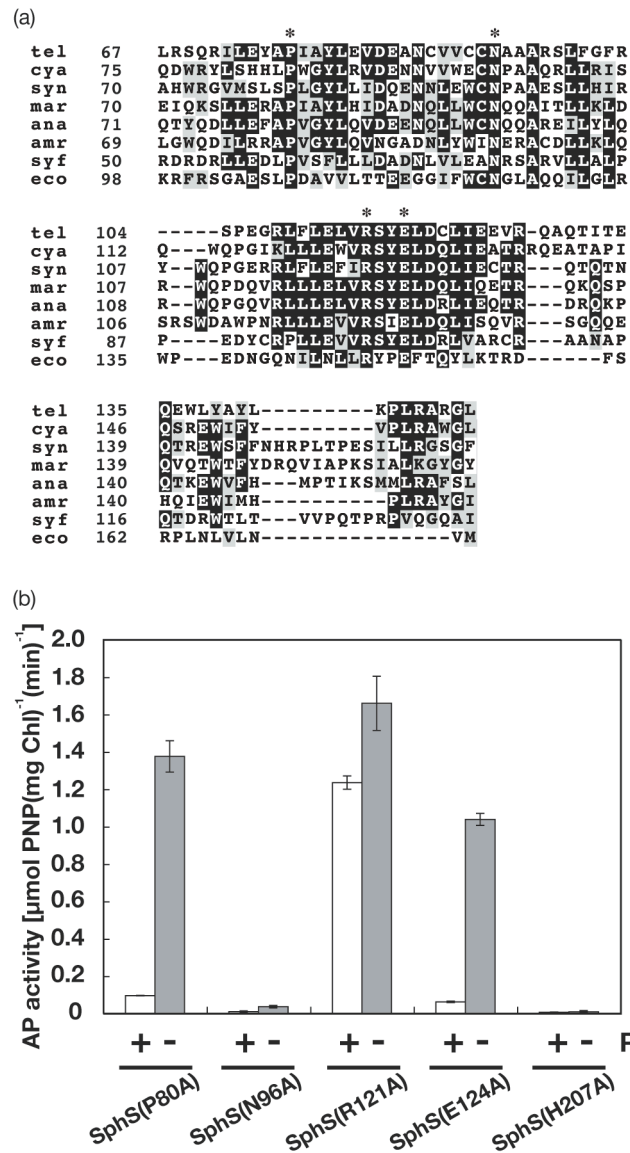


Fig. 4. Effects of point mutations in the PAS domain on the expression of AP activity. Alignment of amino acid sequences of the PAS domain of SphS orthologues (a). tel: *Thermosynechococcus elongates* BP-1 (NP_681715); cya: *Synechococcus* sp. JA-3-3Ab (YP_475742); syn: *Synechocystis* sp. PCC 6803 (NP_442021); mar: *Microcystis aeruginosa* NIES-843 (YP_001660279); ana: *Anabaena* sp. PCC 7120 (NP_488542); amr: *Acaryochloris marina* MBIC11017 (YP_001519542); syf: *Synechococcus elongates* PCC 7942 (YP_400028); eco: *Escherichia coli* K-12 MG1655 (NP_414934). Asterisks indicate the amino acid residues substituted with Ala. Numbers indicate position of amino acid residues. The amino acid alignment was created by ClustalW (Thompson *et al.*, 1994). AP activity in the cells grown under Pi-sufficient (+P, open bar) or Pi-deficient (-P, gray bar) conditions was measured (b). The assays were carried

out three times with cultures grown independently.

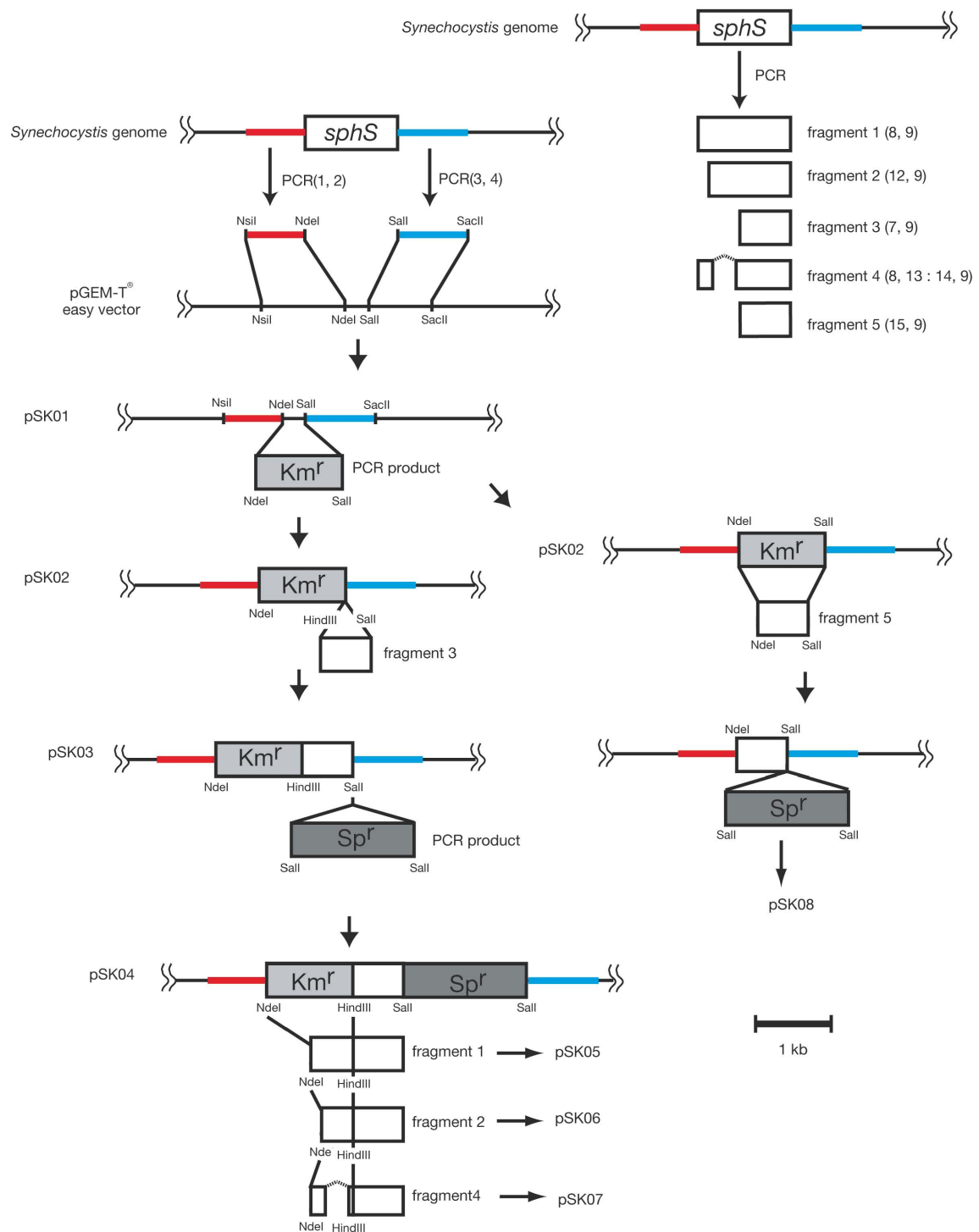


Fig. S1. Construction of *sphS* genes lacking the N-terminal regions. Details are described in materials and methods. The numbers in parentheses corresponds to the primer numbers in Table S1 and were used for amplification of DNA fragments by PCR.

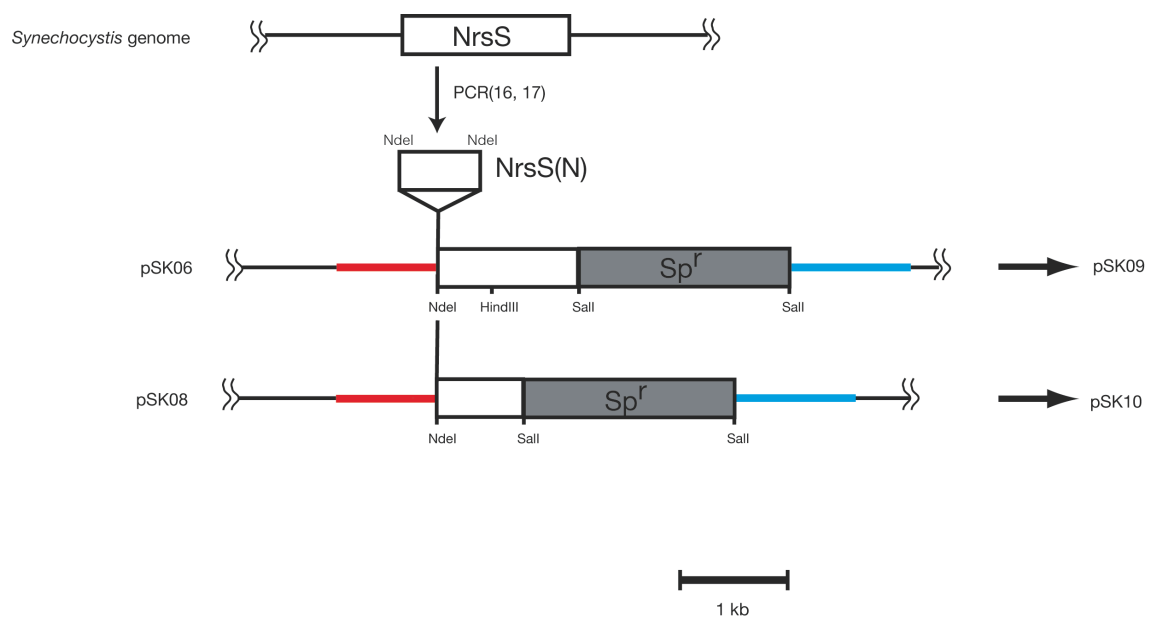
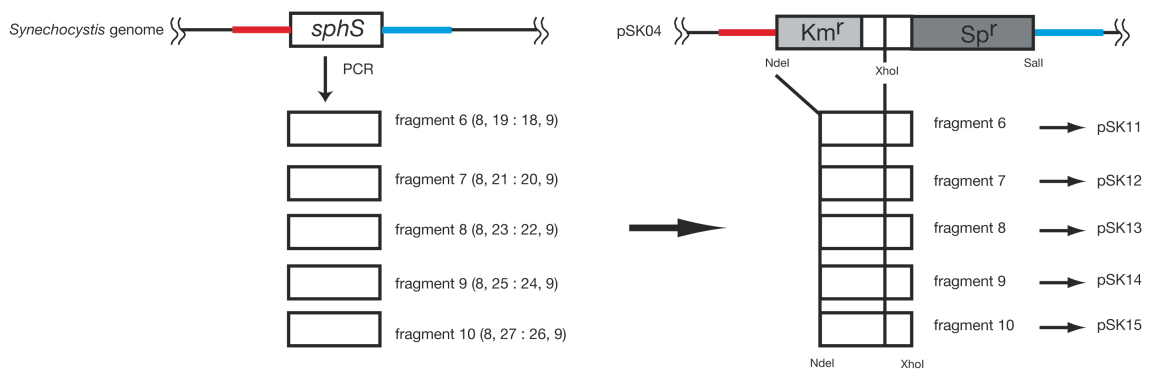


Fig. S2. Construction of *sphS* genes whose hydrophobic region was replaced with that of *NrsS*, a histidine kinase responding to nickel ion. Details are provided in the materials and methods. The numbers in parentheses correspond to the primer numbers in Table S1 and were used for amplification of DNA fragments by PCR.

(a) construction of point-mutated *sphS*



(b) construction of pET-SphS

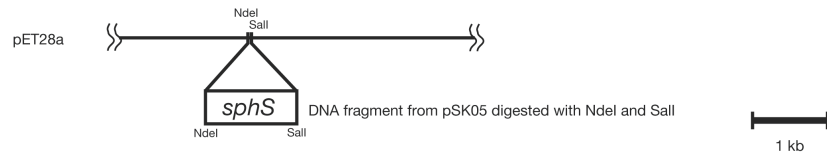


Fig. S3. Construction of *sphS* genes possessing a single amino acid substitution on the PAS domain (a) or His-tagged *sphS* (b). Details are provided in the materials and methods section. The numbers in parentheses correspond to the primer numbers in Table S1 and were used for amplification of DNA fragments by PCR.

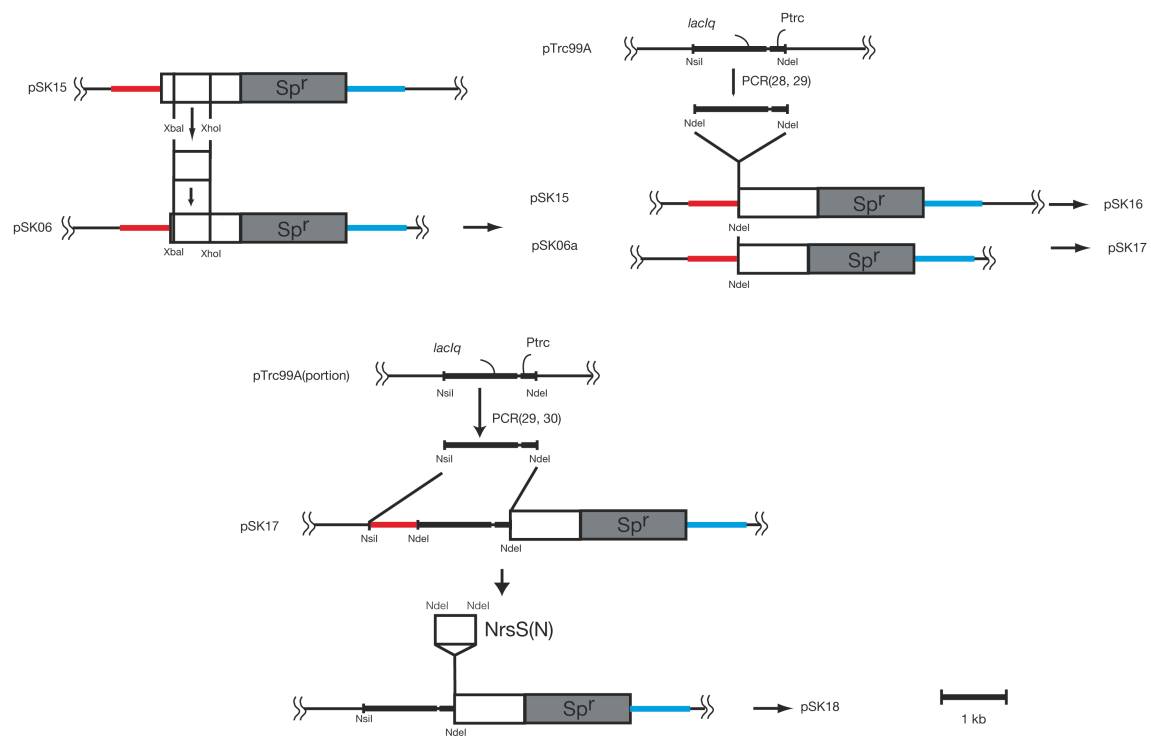


Fig. S4. Construction of plasmids for overexpressing the *sphS* or modified *sphS* genes. Details are provided in the materials and methods section. The numbers in parentheses correspond to the primer numbers in Table S1 and were used for amplification of DNA fragments by PCR.

```
SphS      1  MEIITLAIGGAIGF-GIGAI
NrsS-1   14  --WILGLSFPIALGLVAFSSW
NrsS-2  189  LAFWYALVMGGILTLLGLGVY
```

Fig. S5. Amino acid alignment of hydrophobic regions from SphS and NrsS. Hydrophobic regions of SphS and NrsS were determined by prediction program TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). The amino acid alignment was created by ClustalW.

Table 1. Plasmids used in this study

Plasmid	Description	Reference or source
pGEM [®] -T easy	cloning vector, Ap ^r	Purchased from Promega
pAM1146	source of Sp ^r cassette, Sp ^r	Tsinoremas <i>et al.</i> , 1994
pTrc99a	source of P _{trc} and <i>lacIq</i> , Ap ^r	Amann & Brosius, 1985
pET28a	expression vector, Km ^r	Purchased from Merck KGaA
pSK01	P _{sphS} , Ap ^r	This study
pSK02	P _{sphS} , <i>sphS</i> ⁻ , Ap ^r Km ^r	This study
pSK03	P _{sphS} , Ap ^r Km ^r	This study
pSK04	P _{sphS} , Ap ^r Km ^r Sp ^r	This study
pSK05	P _{sphS} - <i>sphS</i> ⁺ , Ap ^r Sp ^r	This study
pSK06	P _{sphS} - <i>sphS</i> ΔHy ⁺ , Ap ^r Sp ^r	This study
pSK07	P _{sphS} - <i>sphS</i> ΔPAS ⁺ , Ap ^r Sp ^r	This study
pSK08	P _{sphS} - <i>sphS</i> ΔN ⁺ , Ap ^r Sp ^r	This study
pSK09	P _{sphS} -[<i>nrsS</i> (N)- <i>sphS</i> ΔHy] ⁺ , Ap ^r Sp ^r	This study
pSK10	P _{sphS} -[<i>nrsS</i> (N)- <i>sphS</i> ΔN] ⁺ , Ap ^r Sp ^r	This study
pSK11	P _{sphS} - <i>sphS</i> (P80A) ⁺ , Ap ^r Sp ^r	This study
pSK12	P _{sphS} - <i>sphS</i> (N96A) ⁺ , Ap ^r Sp ^r	This study
pSK13	P _{sphS} - <i>sphS</i> (R121A) ⁺ , Ap ^r Sp ^r	This study
pSK14	P _{sphS} - <i>sphS</i> (E124A) ⁺ , Ap ^r Sp ^r	This study
pSK15	P _{sphS} - <i>sphS</i> (H207A) ⁺ , Ap ^r Sp ^r	This study
pSK16	P _{trc} - <i>sphS</i> (H207A) ⁺ , Ap ^r Sp ^r	This study
pSK17	P _{trc} - <i>sphS</i> ΔHy(H207A) ⁺ , Ap ^r Sp ^r	This study
pSK18	P _{trc} -[<i>nrsS</i> (N)- <i>sphS</i> (H207A)ΔHy] ⁺ , Ap ^r Sp ^r	This study
pSK19	His x 6- <i>sphS</i> ⁺ , Km ^r	This study

Supplementary Table 1

Primers used in this study

Primer	Primer	
	number	Sequence (5'–3')
0337uF	1	ATGCATATCCTATAAAGACCGCATCCG
0337uD	2	CATATGCTAGCAGTTGCCTAGTCCGG
0337dF	3	GTCGACAATTCGACCAACAGAAGGTC
0337dR	4	CCGCGGATTGGAGGCAAAGTGGCCAG
kanF	5	CATATGTCTCTTATACACATCTCA
kanR	6	GTCGACACACATCTCAACCCTGAAGC
KinF1	7	AAGCTTTTTCCGATCTGGCC
0337F	8	CATATGGAAATAATTACATTGGC
0337R	9	GTCGACCTAGGGATTTTCATCCATATTCGG
SpeF	10	GTCGACGGATCAATTCCCCTGCTCGC
speR	11	GTCGACTCTCCCAATTTGTGTAGGGC
Δ hyF	12	CATATGGTGCGGCGAGAAATTAATCA
PASuR	13	GGTATCTAGATATTCCGCAC
PASdF	14	GAATATCTAGATACCGGCAAAGTGGCTGTATTTGT
kinF2	15	CATATGGGTAGGGATCAATCTTTTTC
nrsSF	16	CATATGAATACCCGTCGCCTCTT
nrsSR	17	CATATGTTGGTAGGACTGGTAAATGG
P80F	18	TCCCTTTCTGCCCTCGGTTA
P80R	19	TAACCGAGGGCAGAAAGGGA
N96F	20	GAATGGTGTGCTCCTGCGGC
N96R	21	GCCGCAGGAGCACACCATTC
R121F	22	GAGTTTATTGCCTCCTACGA
R121R	23	TCGTAGGAGGCAATAAACTC
E124F	24	CGCTCCTACGCATTGGATCA
E124R	25	TGATCCAATGCGTAGGAGCG
H207F	26	GATCTGGCCGCCGAGTTACG
H207R	27	CGTAACTCGGCGGCCAGATC

trcF	28	AAGGCGAAGCGGCATGCATT
trcR	29	CATATGCTGTTTCCTGTGTG
trcF2	30	AAGGCGAAGCGGCATGCATT

Primer numbers were used to depict the plasmid construction as shown in Supplemental Figures (Fig. S1 – Fig. S4)