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Sequence analysis of the *Microcystis aeruginosa* FACHB-912 phytochrome gene supports positive selection in cyanobacteria

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The phytochrome gene from *Microcystis aeruginosa* FACHB-912, isolated from algal blooms in Taihu Lake in China, was sequenced and found to code for a wavelength transition light receptor protein. The gene was composed of five distinct domains: a PAS fold domain, a GAF domain, a phytochrome domain, a His Kinase A (phosphoacceptor) domain, and a histidine kinase domain. Red-absorbing phytochrome, far-red-absorbing phytochrome, and photoconversion kinetics were monitored via the spectral characteristics of the protein. To detect positive selection, homologous sequences from cyanobacterium phytochrome genes were obtained from GenBank. Potential selective pressure was identified by phylogenetic analysis with maximum likelihood analyses. The frequency of the phytochrome gene being subjected to selective constraints suggests that positive selection is a potentially important mechanism that promotes the evolution of the cyanobacteria phytochrome gene. The sites identified in this study provide targets for further research on the structural-functional role of these residues, and on the correlation with the mechanism of algal blooms.

water bloom, phytochrome, *Microcystis aeruginosa*, positive selection, cyanobacteria, spectral characteristic

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Phytochromes are one of the most important types of photoreceptors. They are interconnected with signal transduction pathways that allow organisms to survive in ambient light environments. Phytochromes are proposed to enhance photosynthetic light capture and help organisms adjust to diurnal and seasonal light fluctuations. In plants, this light-induced phytochrome switch controls several aspects of photo-morphogenesis, including germination, flowering, shade avoidance, and chloroplast development [1,2]. Plant phytochromes have played a key role in evolutionary history [3]. Accumulated data from genome sequencing projects show that phytochromes are not restricted to plants, but are also widely distributed in bacteria, cyanobacteria, and fungi [4].

Plant and bacterial phytochromes have conserved N-terminal chromophore modules with a domain structure, and most bacterial phytochromes have a histidine-kinase module at the C-terminal [5]. Phytochromes mediate responses to red (R) and far-red (FR) light through photoconversion between two stable conformations, an R-absorbing Pr form that is biologically inactive, and an FR-absorbing Pfr form that is biologically active. In this way, phytochromes control recognition via reaction partners of downstream signaling processes [6].

Codon models are used for detecting phylogenetic patterns of positive selection [7], and have been developed into robust methods that can allow selective pressure to vary across sites [8–10]. Selective pressure is measured by the nonsynonymous/synonymous rate ratio, $\omega = dN/dS$, at the protein level. The values of $\omega=1$, <1, and >1 indicate neutral evolution, purifying selection, and positive selection,

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respectively [11]. Individual amino acid sites in a protein are expected to be under different selective pressures and have different underlying ω ratios. The ω ratio is a measure of natural selection acting on the protein. These methods have been used to detect selective pressure in several genes, including the lysozyme genes, tumor suppressor BRCA1 genes, and the phytochrome gene family in angiosperms [12].

Cyanobacteria are oxygenic prokaryotes that can also respond in varied light [13]. Some cyanobacteria, such as *Microcystis aeruginosa*, a surface-growing species, have been known to produce toxins and release them in water [14]. In this study, we cloned and expressed the phytochrome gene of *M. aeruginosa* FACHB-912 (*phy*), elucidated the photochemical properties, and analyzed the sequence characteristics.

1 Materials and methods

1.1 Cyanobacteria and growth conditions

The *M. aeruginosa* FACHB-912 strain was provided by Professor Song Lirong at the Institute of Hydrobiology, Chinese Academy of Sciences. The strain was maintained in BG-11 liquid medium and grown at 30°C in a 12 h∶12 h (light∶dark) cycle at 3000 lx [15]. A daylight lamp acted as a cold light illuminator. To avoid differences in luminous flux, the positions of the conical flasks were randomly changed after every 12 hours of shaking.

1.2 DNA extraction

DNA was extracted from powdered tissue using a lysis buffer (100 mmol L^{-1} Tris-HCl, pH 8.0, 20 mmol L^{-1} EDTA, 1.4 mol L^{-1} NaCl, 3% trimethyl ammonium bromide, and 0.2% (v/v) β-mercaptoethanol). Following centrifugation, the upper aqueous layer was collected and mixed with a phenol∶chloroform∶isoamyl alcohol mixture (25∶24∶1). Finally, nucleic acids in the extracted aqueous phase were precipitated with two volumes of cold ethanol and 1/10 (v/v) of 3 mol L^{-1} Na-acetate at −20°C for 1 h or overnight.

1.3 Plasmid construction

The expression vectors for bilin biosynthesis and *phy* expression were constructed in pCDF Duet-1 (Novagen, Madison, WI, USA), which contains two T7 *lac* promoters. Standard procedures were used for DNA manipulation. All constructs were verified by DNA sequencing.

The genes *hox1* and *pcyA* for bilin biosynthesis were PCR-amplified from *Synechocystis* sp. PCC6803 genomic DNA. Primers used to amplify the *hox1* gene were 5′-ACGCATATGAGTGTCAACTTAGCTTCC-3′ (restriction sites are underlined in all primers) and 5′-ATTGATATCCTAGCCTTCGGAGGTGGC-3′. Primers 5′-TAGATATCAATAAGGAGATATACCATGGCCGTCACTGATTTAAG-3′ and 5′-TGCTCGAGTTATTGGATAACATCAAAT-3′ were used to amplify the *pcyA* gene. The primers used to amplify the *phy* gene from *M. aeruginosa* FACHB-912 were 5′-ACGTCGACATGGCCACCACCGTACAAC-3′ and 5′-ACGCGGCCGCTTAGTTGCCAATGGGGAT-3′. The resulting plasmids, which were constructed to express apo-phytochrome, carried only *phy* genes. To express holo-phytochrome, the plasmids contained three genes: *phy* in one cassette, and *hox1* and *pcyA* in the other. The *hox1* and *pcyA* genes were in tandem array. Protein expression and purification were carried out according to methods described by Landgraf et al. [16].

1.4 Absorption spectrometry

The absorption spectra of the proteins were recorded from 500 to 800 nm using a DU650 spectrophotometer (Beckman Coulter, Indianapolis, IN, USA). The saturation irradiation was set at 670 and 730 nm and came from LED light sources.

1.5 Sequence analysis

The annotated and homologous sequences of the *phy* genes and their respective proteins were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using a BLASTX search against the *phy* gene sequence of *M. aeruginosa* FACHB-912 (GenBank accession number: GU358448). We obtained 10 cyanobacterial *phy* sequences. Attachments were listed as follows (species nomenclature: protein accession number, nucleotide accession number): *Synechocystis* sp. PCC 6803: NP_442237.1, NC_000911.1; *M. aeruginosa* FACHB-912: GU358448, GU358448; *M. aeruginosa* UV027: ACE00425.1, EU723511.1; *M. aeruginosa* PCC 7806: CAO87752.1, AM778953.1; *M. aeruginosa* NIES-843: YP_001656241.1, NC_010296.1; *Cyanothece* sp. PCC 7424: YP_002375377.1, NC_011729.1; *Cyanothece* sp. PCC 7822: ZP_03153288.1, NZ_ABVE01000001.1; *Cyanothece* sp. CCY0110: ZP_01730981.1, NZ_AAXW01000041.1; *Cyanothece* sp. ATCC 51142: YP_001803399.1, NC_010546.1; *Nostoc punctiforme* PCC 73102: YP_001863766.1, NC_010628.1; *Tolypothrix* sp. PCC 7601: AAL76159.1, AF309559.1. Structure domain analysis was performed with Pfam. The retrieved protein and nucleotide sequences were aligned using MUSCLE [17,18] and were checked manually. Coding regions were confirmed using the tranAlign feature of EMBOSS. Where there was little sequence conservation and no improvement in alignment quality, the columns were deleted.

1.6 Phylogenetic analysis

Alignments were carried out using CLUSTALW [19] with default settings, and manually adjusted using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). In this study, we

used *Synechocystis.* sp. PCC 6803 as the outgroup to examine the relationships among the *phy* gene and species. Phylogenetic trees made using the neighbor-joining method were constructed using MEGA 4.0 [20]. Maximum likelihood and maximum parsimony methods were also performed. The reliability of the trees was evaluated using the bootstrap method [21] with 1000 replications.

1.7 Analysis of positive selection

Maximum likelihood models, M0 (one ratio), M1 (nearly neutral), M2 (positive selection), M7 (beta), and M8 (beta & ω), implemented in the program CODEML of PAML version 3.14b [11], were used to test for positive selection. If adaptive evolution occurred at some time points and affected a few amino acids, the above site-specific models might lack power to detect positive selection. Therefore, we also used the branch-site model [12], which allows ω to vary both among sites in the protein and across branches on the tree, and aimed to detect positive selection affecting a few sites among particular lineages. The posterior probability that a particular codon site is positively selected can be estimated using the naïve empirical Bayes (NEB) and the Bayes empirical Bayes (BEB) approach described by the PAML software. The nested models were compared pairwise by the likelihood ratio test (LRT). According to the LRT, twice the log-likelihood difference, $2\Delta l=2(l_1-l_0)$, follows a χ^2 distribution with d$f=P_1-P_0$, where P is the number of free parameters in the model.

2 Results

2.1 Expression and spectral characterization of *phy*

To investigate the spectral characteristics of holo-phytochrome from *M. aeruginosa*, we constructed plasmids carrying *hox1*, *pcyA*, and *phy* genes to express the protein in *Escherichia coli*. The recombinant *E. coli* strains harboring the plasmids carrying *hox1* and *pcyA* turned deep blue-green after induction with isopropyl-β-D-thiogalactopyranoside. In contrast, strains carrying only the *phy* gene displayed no visible color change (Figure S1). The absorption spectra of the purified Phy protein were also compared to evaluate their ability to reversibly photoisomerize. The absorption spectra of Phy showed reversible absorbance shift maxima between 670 and 730 nm when exposed to saturating irradiation in the FR and R light regions (Figure 1). The results of purification of Phy in *M. aeruginosa* are shown in Figure 2. A prominent Coomassie blue-stained band at 86 kD was observed, which is consistent with the calculated molecular weight of Phy.

2.2 Domain analysis

In phytochromes from plants and cyanobacteria that bind

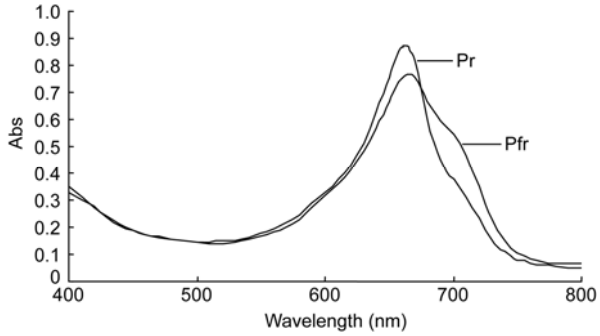

Figure 1 Photo-conversion absorption spectra of Phy (*M. aeruginosa* FACHB-912). Pfr, Pfr-enriched form obtained after illumination with red light; Pr, form obtained after illumination with far-red light. Because of spectral overlaps of the Pr and Pfr states of Phy, the photo-conversion from Pr to Pfr is more than 50% complete.

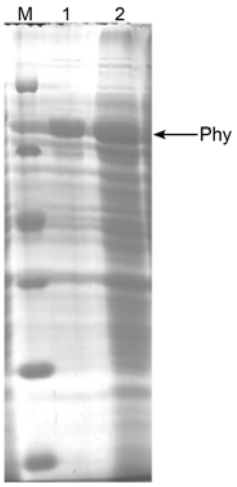

Figure 2 SDS-PAGE analysis. Lane 1, Phy purified by Histrap HP Ni^{2+} affinity column (GE Amersham Biosciences). Lane 2, total proteins expressed in *E. coli*. M, molecular mass standard, from the top: 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 kD.

phycocyanobilin (PCB), the chromophore is bound to a fully conserved cysteine in the GAF domain, which is named after the cGMP-regulated cyclic nucleotide phosphodiesterases, adenylyl cyclases, and bacterial transcription factor FhlA domains [22]. In biliverdin (BV)-binding bacterial phytochromes, the binding site is located in the Per-Arnt-Sim (PAS) domain at the N-terminus of the protein [23,24]. Thus, we can conclude that members of the phytochrome superfamily have a similar protein organization that consists of a signature N-terminal chromophore-binding domain that autocatalytically binds an assortment of bilin chromophores, followed by a divergent C-terminal module that is involved in signal transduction and homodimerization (Figure 3) [25]. Phys, Cphs, and Bphs use the bilins 3E-phytochromobilin, PCB, and BV as chromophores, respectively [26,27].

2.3 Phylogeny of *phy* gene

The resulting consensus tree shows that the *phy* genes were

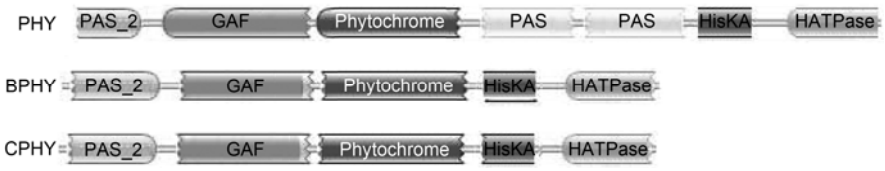

Figure 3 The compared structures of CPHY (*M. aeruginosa* FACHB-912), BPHY (*Rhodopseudomonas palustris* CGA009, RpPhyB4) and PHY (*Arabidopsis thaliana,* PhyA). PAS, Per-Arnt-Sim; HisKA, His kinase A (phosphoacceptor) domain; HATPase, Histidine kinase domain.

separated into five clades with *Synechocystis* sp. PCC 6803 as the outgroup (Figure 4). *M. aeruginosa* strains shared the same clade with high similarity. The *phy* gene of *M. aeruginosa* FACHB-912 had more affinity with *M. aeruginosa* NIES-843 among the clade. Also, *Cyanothece* sp. PCC 7424 and *Cyanothece sp.* PCC 7822 were most closely related to *M. aeruginosa*. Phylogenies constructed with different methods (maximum parsimony and maximum likelihood) showed similar topologies. The phylogeny of *phy* genes in bacteria and angiosperms has been well characterized in previous studies [12,28].

2.4 Selective constraints in evolution of *phy*

The one-ratio model (M0) yielded an estimate of $\omega = 0.0741$ (Table 1), indicating strong selective constraints in *phy*. We applied the site-specific likelihood models [8,10] to the *phy* gene data (Table 1), which assume variable selective pressures among sites but no variation among branches in the phylogeny. No positively selected sites were identified using M1, M2 and M7, whereas only a small number of sites were found to be under positive selection using M3 ($P > 0.80$) and M8 ($P > 0.90$).

We used three pairs of models, forming three LRTs: M1 (neutral) and M2 (selection), M0 (one ratio) and M3 (discrete), and M7 (beta) and M8 (beta & ω) [8,10]. Because model M2 (selection) is an extension of M1 (neutral), the two models can be compared using an LRT. The test statistic is $2\Delta l$ =((−13799.705043)−(−13799.705043))=0, with $P >$ 0.995 and d$f = 2$. Therefore, model M2 is not significantly better than M1. The discrete model (M3) was significantly better than the one-ratio model; the test statistic is $2\Delta l$ = ((−13621.256427)−(−13920.743605))=298.51, and $P < 0.001$ with d$f = 9$. This result means that the residues 238E and 667T are significant despite the low posterior probabilities. However, the difference between M7 and M8 is not statistically significant for $2\Delta l$ = ((−13622.134102)−(−13624.312405))= 2.18, with $P > 0.25$ and d$f = 2$. We note that the M0-M2 comparison is more of a test of variability in the ω ratio among sites rather than a rigorous test of positive selection. However, the model of a single ω for all sites is probably wrong in every functional protein, so there is no need for further testing. The high level of sequence similarity between the *phy* genes may be the reason for the non-significant results of the M1-M2 and M7-M8 comparisons, resulting in a lack of power of the LRTs.

The branch-site models implemented in this study were also applied to the *phy* gene, with branch # from Figure 3 considered as the foreground branch and all other branches in the tree as background branches. Model A can be compared with the site-specific model M1 (neutral); the LRT statistic is $2\Delta l$ = ((−13784.438258)−(−13799.705043))=15.27, with $P < 0.005$ and d$f = 2$, showing model A is significantly better than model M1. The comparison between Model B and the site-specific model M3 (discrete) gave $2\Delta l$ = ((−13637.858327)−(−13799.705043))=161.85, with $P < 0.001$ and d$f = 2$. This also reveals the reliability of Model B.

The sites under positive selection along branch # in the

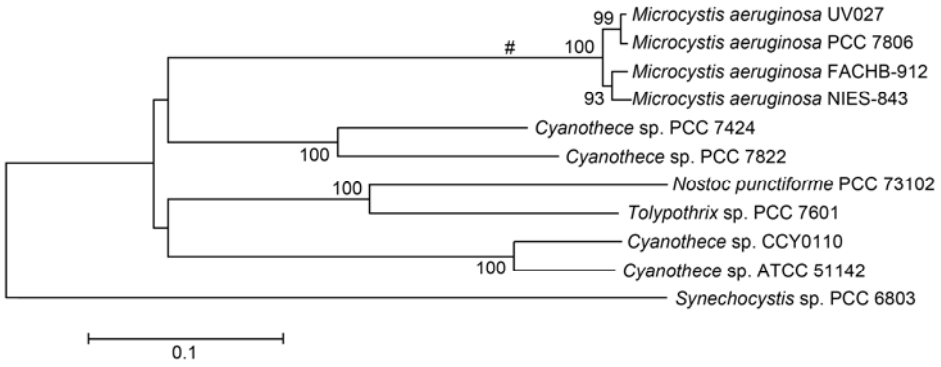

Figure 4 The phylogenetic tree of the phytochrome gene (*phy*) in 11 cyanobacteria species. *Synechocystis* sp. PCC 6803 was the outgroup. # represents the internal branch ancestral to *M. aeruginosa* that had the ratio ω_1, while all other branches (with the default label #0) had the background ratio ω_0. The *M. aeruginosa* lineages were proposed to be under positive selection. The bootstrap values lower than 50% were cut off.

Table 1 Parameter estimates and log-likelihood values for phytochrome under different models[a)]

Model	*P*	ln*L*	Estimates of parameter	Positively selected site
M0: one-ratio	1	−13920.743605	ω_0=0.0741	None
Site-specific models				
M1: neutral	1	−13799.705043	P_0=0.91484 (P_1=0.08516), ω_0=0.06640, ω_1=1.0000	NA
M2: selection	3	−13799.705043	P_0=0.91484, P_1=0.05238, P_2=0.03279, ω_0=0.06640, ω_1=1.0000, ω_2=1.0000	None
M3: discrete	10	−13621.256427	ω=0.0913	238E 667T (at *P*>0.80)
M7: beta	2	−13624.312405	*P*=0.46160, *q*=4.61851	None
M8: beta&ω	4	−13622.134102	P_0=0.99653, *P*=0.47595, *q*=4.95405, P_1=0.00347, ω=2.14228	238E 667T (at *P*>0.90) 47R 111A 227E (at *P*>0.50)
Branch-site models				
Model A	3	−13784.438258	P_0=0.87860(background ω=0.06349), P_1=0.07450 (P_2+P_3=0.04690), ω=24.85804	148R 279R 351A 390T 604L (at *P*>0.95) 11S 113Q 279R 455I 604L 626V 736V (at *P*>0.90) 155N 189I 202A 220Q 316R 343N 378V 452G 473S 514R 517R 598N 599T 603N 614K 636K 660T 755A (at *P*>0.50)
Model B	5	−13637.858327	P_0=0.43199 (background ω=0.01748), P_1=0.26984 (P_2+P_3=0.29817), ω=0.19837	None

a) *P* is the number of the ω distribution. Sites potentially under positive selection were identified using *M. aeruginosa* FACHB-912 sequence as the reference.

tree are 11S, 113Q, 148R, 238E, 279R, 351A, 390T, 455I, 604L, 626V, 667T, and 736V, according to high posterior probabilities. The following 21 sites along the branch: 47R, 111A, 155N, 189I, 202A, 220Q, 227E, 316R, 343N, 378V, 452G, 473S, 514R, 517R, 598N, 599T, 603N, 614K, 636K, 660T, and 755A have relatively low posterior probabilities and can also represent the potentially strong positive selection in *phy* genes.

3 Discussion

The *phy* gene of *M. aeruginosa* FACHB-912 shares many similarities with the corresponding gene in other plants and bacteria, including the composition of the individual domains in the resulting protein structure that are involved in the chromophore-binding GAF, Phy and histidine kinase [23]. These domains are essential and sufficient for Pr/Pfr conversion [24]. PAS domains are signaling modules that survey changes in light, oxygen, and the overall energy level [29]. As mentioned above, Phy contains the C-terminal histidine kinase module, which includes light-regulated histidine kinases. The dramatic reduction from R to FR is the same as the spectral range covered by the absorption of the Pr/Pfr forms of BPHY [30].

Given that organisms are able to adapt to the surrounding environment and monitor environmental changes such as temperature and light conditions, natural selection plays a key role in selecting for such changes in their evolution. Light is of critical importance to plant metabolism, and plants employ a number of mechanisms to detect light. Phytochromes are the best-characterized photosensors. Previous studies have revealed positive selection was involved in the functional divergence of the PhyACF and PhyBDE subfamilies. This caused adaptive changes at certain amino acid sites along branches immediately after the gene duplications that separated the A from the C–F and that separated the B–D and the E, whereas purifying selection suppressed the divergence in each group [12]. Moreover, positive selection at a handful of sites, rather than relaxation of selective constraints, has played a major role in the evolution of PhyA in early angiosperms [31]. Because light signals differ among latitudes and altitudes, the functions of phytochromes should be targeted towards adaptation to the local light environment. This hypothesis is supported by recent studies in *Arabidopsis thaliana*, which showed that amino acid substitutions in PHYA, PHYB, and PHYC may be responsible for natural variation in plant flowering and growth response [32–34]. Another study of local adaptation in PhyB2 in *Populus tremula* has also suggested this hypothesis [35,36]. An overall low level of nucleotide diversity in *phy* genes suggests that the evolution of phytochromes is constrained by purifying selection within *Cardamine nipponica*, which is consistent with previous findings on the molecular evolution of phytochromes [37–39]. The exception is that specific high levels of nonsynonymous substitutions in *phyE* in *C. nipponica* may be attributable to the elevated nonsynonymous substitutions between haplotypes in central and northern Japan [3]. We can conclude that the evolution is compatible with both positive selective pressure and relaxed evolutionary constraint.

Three homologous *M. aeruginosa* strains, including *M. aeruginosa* FACHB-912, are distributed in different geographical locations, so they can be expected to adapt to lo-

cal conditions. Positive selection represents the effects of natural selection pressure and can lead to apparent substitution rate heterogeneity and genetic adaptation. Given the results obtained, which showed a high frequency of *phy* selective constraints in cyanobacteria strains, and the biological relevance related to the regions, we conclude that positive selection may be a potentially important mechanism that generates genetic variation in the phytochromes of cyanobacteria.

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