

MarR-type Transcriptional Regulator ChlR Activates Expression of Tetrapyrrole Biosynthesis Genes in Response to Low-oxygen Conditions in Cyanobacteria*[§]

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Background: Oxygen is required for three enzymatic steps in tetrapyrrole biosynthesis.

Results: A transcriptional regulator gene was found by genome resequencing of a mutant of *Synechocystis* sp. PCC 6803.

Discussion: Sll1512 (ChlR) is a MarR-type transcriptional regulator involved in induction of genes in response to low-oxygen conditions.

Significance: Maintaining the chlorophyll level constant under hypoxia is important for cyanobacteria to survive.

Oxygen is required for three enzyme reactions in chlorophyll and bilin biosynthesis pathways: coproporphyrinogen III oxidase (HemF), heme oxygenase (HO1), and Mg-protoporphyrin IX monomethylester cyclase (ChlA_I). The cyanobacterium *Synechocystis* sp. PCC 6803 has alternative enzymes, HemN, HO2, and ChlA_{II}, to supply chlorophyll/bilins even under low-oxygen environments. The three genes form an operon, *chlA_{II}-ho2-hemN*, that is induced in response to low-oxygen conditions to bypass the oxygen-dependent reactions. Here we identified a transcriptional regulator for the induction of the operon in response to low-oxygen conditions. A pseudorevertant, Δ *ho1R*, was isolated from a HO1-lacking mutant Δ *ho1* that is lethal under aerobic conditions. Δ *ho1R* grew well even under aerobic conditions. In Δ *ho1R*, HO2 that is induced only under low-oxygen conditions was anomalously expressed under aerobic conditions to complement the loss of HO1. A G-to-C transversion in *sll1512* causing the amino acid change from aspartate 35 to histidine was identified as the relevant mutation by resequencing of the Δ *ho1R* genome. Sll1512 is a MarR-type transcriptional regulator. An *sll1512*-lacking mutant grew poorly under low-oxygen conditions with a remarked decrease in Chl content that would be caused by the suppressed induction of the *chlA_{II}* and *hemN* genes in Chl biosynthesis under low-oxygen conditions. These results demonstrated that Sll1512 is an activator in response to low-oxygen environments and that the D35H variant becomes a constitutive activator. This hypothesis was supported by a gel shift assay showing that the Sll1512-D35H variant binds to the DNA fragment upstream of the operon. We propose to name *sll1512 chlR*.

Cyanobacteria are photosynthetic prokaryotes that adapt and thrive in a wide variety of environments on the Earth (1). They perform oxygenic photosynthesis like plants by extracting electrons from water with generation of oxygen as a byproduct. Cyanobacteria also use oxygen for respiration to produce energy in the dark (2). The oxygen levels greatly change in the field environment from hyperoxic to almost anaerobic conditions (3, 4). Even in aerobic environments, cyanobacterial cells are exposed to anaerobic conditions in the night on a daily basis because oxygen is quickly consumed by respiration in the cells (5, 6). Thus, adaptation of anaerobiosis is important for cyanobacteria to survive.

Chl and bilin are important tetrapyrrole pigments in photosynthesis. Their biosynthetic pathways from glutamate to protoporphyrin IX is the same as that of heme (7, 8). The Chl-specific pathway starts from the insertion of a magnesium ion into protoporphyrin IX by Mg-chelatase. Protoporphyrin IX is converted to heme by the insertion of ferrous ion catalyzed by ferrochelatase. Bilin biosynthesis starts from the oxygen-dependent cleavage of heme catalyzed by heme oxygenase (HO)². Photosynthetic organisms, including cyanobacteria, should have elaborate mechanisms to regulate the biosynthetic pathways of Chl and bilin to avoid photooxidative damage caused by the accumulation of free pigments and their intermediates. However, in plants, the mechanisms largely remains to be solved while some plausible mechanisms are proposed (9, 10).

In the biosynthetic pathways of Chl and bilin pigments, there are at least three oxygen-dependent reactions (7, 8). One is coproporphyrinogen III oxidation catalyzed by oxygen-dependent coproporphyrinogen III oxidase. In this reaction, oxygen is required for oxidative decarboxylation (11). The second is the heme cleavage reaction to form biliverdin IX α catalyzed by HO (12, 13). Third is the fifth ring (E-ring) formation of chlorophyll, catalyzed by Mg-protoporphyrin IX monomethylester cyclase (ChlA_I) (14). In this reaction, Mg-protoporphyrin IX monom-

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[§] This article contains supplemental Figs. S1–S5 and Tables S1 and S2.

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² The abbreviations used are: HO, heme oxygenase; Chl, chlorophyll; PC, phycocyanin; PCC, Pasteur culture collection; GT, glucose-tolerant; KEGG, Kyoto encyclopedia of genes and genomes.

ethyl ester is converted to protochlorophyllide through oxygenation of the C13-methylpropionate and ring closure reactions with the incorporation of an oxygen atom as the oxo group at the C13¹ position. In the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), these oxygen-dependent reactions are bypassed by three sets of enzymes (12). An analogous coproporphyrinogen III oxidase, HemN, catalyzes the reaction in an oxygen-independent manner instead of HemF (11). Low-oxygen-specific isoforms, HO2 and ChlA_{II}, operate together with the constitutive isoforms, HO1 and ChlA_I, respectively (12, 14). The three low-oxygen-specific genes form a small operon, *chlA_{II}-ho2-hemN*, and are induced in response to low-oxygen conditions. This operon is also conserved in another cyanobacterium *Synechococcus* sp. PCC 7002 (*Synechococcus* 7002) and the transcription of the operon is also induced by low-oxygen conditions as well as *Synechocystis* 6803 (15). However, the low-oxygen induction mechanism is still elusive in cyanobacteria. Recently Summerfield *et al.* (16) reported that Hik31-histidine kinase plays a role as a negative regulator for expression of some genes in response to low oxygen in *Synechocystis* 6803. However, because the expression profile of the *chlA_{II}-ho2-hemN* operon is not altered in the ΔHik31 mutant, this operon is regulated by a Hik31-independent pathway that has remained unknown.

Here we report a MarR-type transcriptional regulator Sll1512 involved in the low-oxygen induction of the *chlA_{II}-ho2-hemN* operon through genome resequencing of a mutant with a Next Generation Sequencer SOLiD. Sll1512 would play a role as an activator for transcription of the operon in response to low-oxygen environments.

EXPERIMENTAL PROCEDURES

Cyanobacterial Strains and Growth Conditions—*Synechocystis* sp. PCC 6803 and its derivative strains used in this study were cultivated in BG-11 medium as described (12). To prepare cells grown under aerobic and low-oxygen conditions, we incubated agar plates in air and in an anaerobic jar (BBL GasPak anaerobic systems, BD Biosciences; gas-generating kit anaerobic system, Oxoid, Basingstoke, Hants, UK), respectively. Agar plates under both conditions were illuminated at light intensity of 50 μmol_{photon} m⁻²s⁻¹ (FLR40SW, Hitachi, Tokyo, Japan).

In the process of genome resequencing, our wild-type “YF” was found to have a genome sequence closer to PCC strains (PCC-N and PCC-P; 17) rather than GT strains (GT-Kazusa, GT-S, and GT-I 17,18) because three SNPs in the YF genome were identical to those of PCC strains (supplemental Table S1).

Determination of Chl and PC Contents—The concentration of Chl in the 90% methanol extracts from the cells was determined by absorption at 665 nm (model V-550, JASCO, Hachioji, Japan) (19). The amount of Chl was normalized by the optical density at 730 nm (A₇₃₀; UV1700, Shimadzu, Kyoto, Japan) of the cell suspension used for the extraction. PC contents were determined as described (12). The protein concentration was determined by a dye-binding assay (protein assay, Bio-Rad) using bovine serum albumin as a standard.

Preparation of RNA and RT-PCR—Cells were grown on the agar plates for 7 days, collected, and harvested by centrifugation

at 4 °C. RNA preparation and the following RT-PCR procedures were described previously (14). The isolated total RNA was used for the synthesis of cDNA with ReverTra Ace (Toyobo, Osaka, Japan) and reverse primers for RT-PCR (supplemental Table S2) according to the manufacturer's manual. Thus, obtained cDNA fraction was used as the template for PCR amplification with the specific primers (supplemental Table S2).

Solid DNA Sequencing—Sequencing runs were done using cyclic ligation sequencing on a SOLiD analyzer (Invitrogen). Ten micrograms of purified genomic DNA of wild-type, Δ*ho1*, and Δ*ho1R* were sheared into ~125-bp short fragments with the Covaris S2 system (Covaris, Woburn, MA). The sheared DNA fragments were blunted by Next NR enzyme (New England Biolabs, Beverly, MA) and then purified using a Pure Link PCR purification kit (Invitrogen). The purified DNA fragments were ligated to short SOLiD P1 and P2 adapters (supplemental Table S2) by T4 Ligase (Invitrogen). Adapter-ligated DNA was purified using the Pure Link PCR purification kit. These DNA fragments were nick-translated and amplified by PCR (cycle number 3, supplemental Table S2) and then purified using the Pure Link PCR purification kit. The purified PCR products were separated by SOLiD size selection gel (2% agarose gel, Invitrogen) and collected the fragments corresponding 150- to 200-bp DNA. These DNA libraries were quantified by quantitative PCR using a SOLiD TaqMan probe.

In preparation for sequencing, the DNA fragments were clonally amplified by emulsion PCR using beads with P1 primer covalently attached to the surface. Emulsions were broken with butanol, and emulsion PCR beads were enriched by hybridization with P2-coated capture beads (Life Technology). These beads were added dTs at the 3' end in the presence of terminal transferase to connect on the slide glass. About 60 million beads were deposited onto one-fourth of a glass surface of a 25 mm × 75 mm SOLiD slide. The slide was loaded onto a SOLiD instrument, and the 50-base sequences were obtained according to the manufacturer's protocol.

SNP Analysis—DNA sequence data (csfasta) were analyzed using Takeru II (NABE International, Tsukuba, Japan). Sequence data were mapped to the genome sequence of *Synechocystis* 6803 obtained from KEGG using “Map Data” of BioScope (v. 1.2). From them, we gained SNPs data using SNPs (supplemental Fig. S2).

Transformation Assay for Identification of SNPs—The *sll1512* (2,065 bp) and *slr0887* (2,098 bp) fragments containing an SNP site were amplified by PCR using the genomic DNA of Δ*ho1* and Δ*ho1R* by a standard thermal cycle (KOD-plus DNA polymerase, Toyobo). The amplified DNA fragments were separated by 1% agarose gel and purified by Wizard SV gel and PCR clean-up kit (Promega, Madison, WI). During preparation of the *sll1512* fragment, we found another nonspecific fragment with almost the same size as the *sll1512* fragment. We removed it by digesting a mixture of these fragments with BstXI. The resultant 1,942-bp fragment was purified by agarose gel electrophoresis. The cell suspension of Δ*ho1* (300 μl, A₇₃₀ = 1) was spread on BG-11-agar plate containing kanamycin. The DNA fragments (2–60 ng/μl, 5 μl) were spotted on the plate. The

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plate was incubated under low-oxygen conditions for 3 days and then transferred under aerobic conditions for 2 weeks.

Construction of Plasmids for *sll1512* Disruption and Transformation—Plasmids for targeted gene disruption were constructed as follows (Fig. 3A, supplemental Table S2). A DNA fragment (2,338 bp) containing *sll1512* was amplified by PCR from genomic DNA of *Synechocystis* 6803 with the primer pairs (supplemental Table S2) by a standard thermal cycle (KOD-plus DNA polymerase). The amplified DNA fragments were cloned into the BamHI site of pUC118. The recombinant plasmid carrying *sll1512* was digested by HpaI and BsmI (Fig. 3A). A spectinomycin-resistant cartridge amplified from pJN3 (20) was inserted into the blunted HpaI and BsmI sites to construct the *sll1512*-disrupted plasmid. *Synechocystis* 6803 was transformed with the plasmid constructed as described (12).

Overexpression of *Sll1512* in *Escherichia coli* and Purification—The plasmids for overexpression of Sll1512 (wild type) and the D35H variant were constructed as follows. The BstXI-digested 1,942-bp fragments from $\Delta ho1$ and $\Delta ho1R$ that were prepared for the transformation assay were used as the template to amplify the coding regions of Sll1512 and Sll1512-D35H, respectively, by PCR in a standard thermal cycle (supplemental Table S2). After digestion with BsaI, the amplified DNA fragments were cloned into the BsaI site of pASK-IBA5plus (IBA, Göttingen, Germany) to form pASK5-Sll1512 and pASK5-Sll1512D35H. The N-terminal sequence of Strep-tagged Sll1512 is MASWSHPQFEKGA followed by the second amino acid residue (Glu) of the native sequence. *E. coli* JM105 transformants harboring the plasmids were cultivated in LB (1% Trypton, 0.5% Yeast extract, 1% NaCl) medium (5 ml) containing 100 $\mu\text{g/ml}$ ampicillin for 6 h with vigorous shaking at 37 °C. Overexpression of the protein was induced upon the addition of anhydrotetracycline (final 200 ng/ml) 2 h after inoculation. *E. coli* cells harvested by centrifugation were washed in 1 ml of 0.9% (w/v) NaCl, resuspended in lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM PMSF) and disrupted by sonication (Sonifier model 250; Branson, Danbury, CT). The soluble fractions were obtained by centrifugation of the sonicates (15,000 rpm, 4 °C, 10 min). Affinity purification with Strep-Tactin was carried out as described (21).

Gel Shift Assay—Two DNA fragments upstream of *chlA_{II}* and *psbA1* (312 bp from the position -1 to -312 of *chlA_{II}*, 297 bp from the position -1 to -297 of *psbA1*) were amplified by PCR from genomic DNA of *Synechocystis* 6803 (supplemental Table S2). DNA fragments were labeled at both termini with ³²P and used as the probe for gel retardation assays. Gel retardation assays were performed essentially as described (22). Proteins were incubated with probes (0.1 nM) in 20 μl of binding buffer (20 mM HEPES-KOH, pH 8.0, 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 60 mM KCl, 12% glycerol, 0.1 mg ml⁻¹ BSA, 0.05 mg ml⁻¹ poly(deoxyinosinic-deoxycytidylic) acid sodium salt) for 20 min at 30 °C. The mixtures were separated by a native 4% polyacrylamide gel. Gels were dried, and the signals were detected by autoradiography.

RESULTS

Isolation of a Pseudorevertant $\Delta ho1R$ and Characterization—Previously, we isolated a *ho1*-deficient mutant, $\Delta ho1$, that cannot survive under aerobic conditions but can grow under low-oxygen

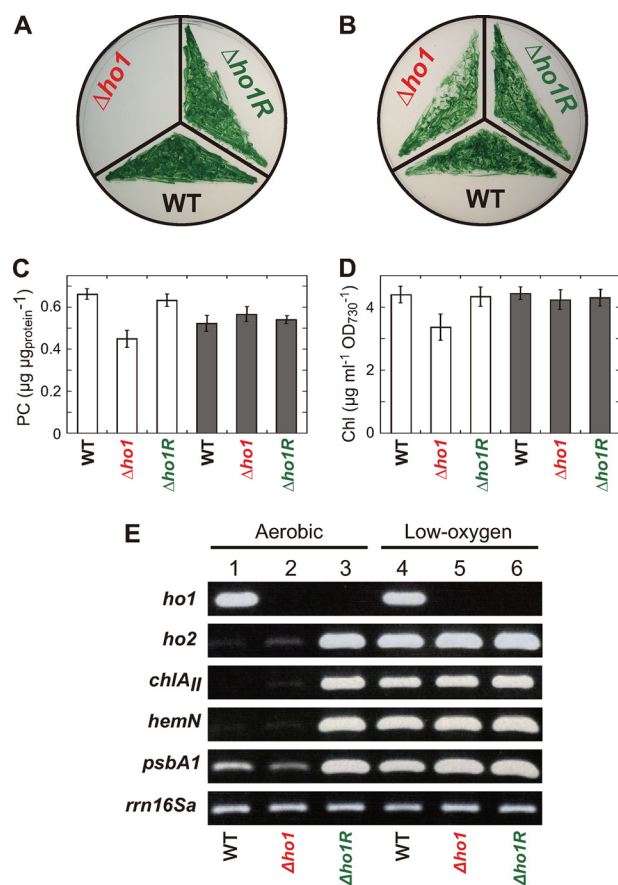


FIGURE 1. Characterization of a pseudorevertant $\Delta ho1R$. Comparison of photoautotrophic growth (A and B) and PC (C) and Chl (D) contents. Cells grew under aerobic (A) and low-oxygen (B) conditions for 7 days. PC (C) and Chl (D) were extracted from cells grown under low-oxygen conditions for 5 days followed by 2-day aerobic incubation (white bars) and from cells grown under low-oxygen conditions on the agar plates for 7 days (gray bars). The data are mean \pm S.D. for three independent experiments. E, RT-PCR analysis for the transcript levels of *ho1*, *ho2*, *chlA_{II}*, *hemN*, and *psbA1*. Total RNA was isolated from the wild-type (lanes 1 and 4), $\Delta ho1$ (lanes 2 and 5), and $\Delta ho1R$ (lanes 3 and 6) cells. Aerobic samples (lanes 1-3) were grown under low-oxygen conditions for 5 days and then transferred under aerobic conditions and grown for 2 days. Low-oxygen samples (lanes 4-6) were grown under low-oxygen conditions for 7 days. The housekeeping gene *rrn16Sa* was used as a control. Cycle numbers of RT-PCR were 6 and 25 for *rrn16Sa* and the others, respectively.

conditions (12). A single colony survived on an agar plate incubated under aerobic conditions where all other $\Delta ho1$ cells died. We picked up the colony as a pseudorevertant " $\Delta ho1R$ " for further study. We confirmed that $\Delta ho1R$ could grow under aerobic conditions where the original strain $\Delta ho1$ could not (Fig. 1A). The growth of $\Delta ho1R$ under low-oxygen conditions was equal to that of the wild type (Fig. 1B).

PC and Chl contents in $\Delta ho1R$ were determined (Fig. 1, C and D). PC is a dominant phycobilisome protein of *Synechocystis* 6803. The PC and Chl contents in aerobically incubated $\Delta ho1$ were significantly decreased as shown previously (12). The levels of PC and Chl in $\Delta ho1R$ were the same as those of the wild type under aerobic conditions.

After we confirmed the absence of *ho1* in $\Delta ho1R$, we examined the transcript levels of the low-oxygen inducible operon consisting of *chlA_{II}*, *ho2*, and *hemN* in $\Delta ho1R$ (11, 12, 14) by RT-PCR (Fig. 1E). Surprisingly, the *ho2* transcript level in aerobically grown $\Delta ho1R$ was almost comparable with that in

$\Delta ho1R$ grown under low-oxygen conditions (Fig. 1E, lane 3), which was in contrast to the expression profiles of $ho2$ in the wild type and $\Delta ho1$. This change in the transcript levels was also observed in the $chlA_{II}$ and $hemN$. In $\Delta ho1R$, all three genes in the operon were expressed in a constitutive manner in both aerobic and low-oxygen conditions. This result suggested that the constitutive expression of $ho2$ restores the defect of $ho1$ to allow growth under aerobic conditions. This is consistent with the observation of the $ho2$ overexpression by an artificial promoter restored the $\Delta ho1$ growth defect (12). Summerfield *et al.* (23) reported five gene clusters, including the $chlA_{II}$ operon, that are induced in response to low-oxygen conditions in *Synechocystis* 6803. We examined whether the expression patterns of 10 representative genes ($chlG$, por , $ctpA$, $hoxE$, $flv4$, $psbA1$, ppc , $ppsA$, $cpcG2$, and $atpF$) are altered in $\Delta ho1R$ by RT-PCR (supplemental Fig. S1 and Fig. 1E). The mRNA levels of most genes except for $flv4$ and $psbA1$ did not change significantly between wild-type cells grown under aerobic and low-oxygen conditions, and the expression profile in $\Delta ho1R$ was almost the same as that in the wild type. The $flv4$ transcript was not detected in any cells grown under low-oxygen conditions. $psbA1$ encoding an isoform of the D1 protein (D1') of photosystem II showed a clear low-oxygen induction profile in the wild type and $\Delta ho1$, and the profile was changed to a constitutive one in $\Delta ho1R$ like the $chlA_{II}$ operon (Fig. 1E).

Genome Resequencing of $\Delta ho1R$ and Identification of the Mutation Site—To identify the relevant mutation(s) responsible for the growth restoration of $\Delta ho1R$, we carried out whole genome resequencing of $\Delta ho1R$, $\Delta ho1$, and the wild type by a next-generation sequencer, SOLiD. Comparison with the Genome Database of Cyanobacteria, CyanoBase, revealed 12 SNPs that were common in all the three strains (supplemental Table S1). Six of these 12 SNPs were recently reported to be sequence errors in the data base (18), three are identical SNPs found in PCC strains (17), and the remaining three appear to be unique to our wild-type strain YF. In addition to the common 12 SNPs, we found two SNPs unique to $\Delta ho1R$. One was a G-to-C transversion (at 462,452) in $sll1512$ that causes an amino acid substitution from Asp-35 to His, and the other was a C-to-A transversion (at 1,153,565) in $slr0887$ causing an amino acid replacement of Pro-92 to Thr (supplemental Fig. S2 and Table S1).

We performed a transformation analysis to identify which of the two $\Delta ho1R$ -specific SNPs is responsible for the growth recovery of $\Delta ho1$. DNA fragments of about 2 kb containing the SNPs in $sll1512$ and $slr0887$ were amplified by PCR from the genomes of $\Delta ho1$ and $\Delta ho1R$ to transform $\Delta ho1$. Many colonies appeared on only one sector transformed with the $sll1512$ fragment from $\Delta ho1R$, whereas no colonies appeared on the other three sectors (Fig. 2). This result clearly indicated that the D35H mutation in $sll1512$ is responsible for the recovery of growth in $\Delta ho1$ under aerobic conditions.

Isolation of an $sll1512$ -lacking Mutant and Phenotype Analysis— $sll1512$ encodes a protein with 135 amino acid residues (Fig. 3A). Although Sll1512 is annotated as a hypothetical protein in the cyanobacterial genome databases CYORF and CyanoBase, they suggested that 11 cyanobacteria carry probable orthologs of $sll1512$ (supplemental Fig. S3). A BLAST search

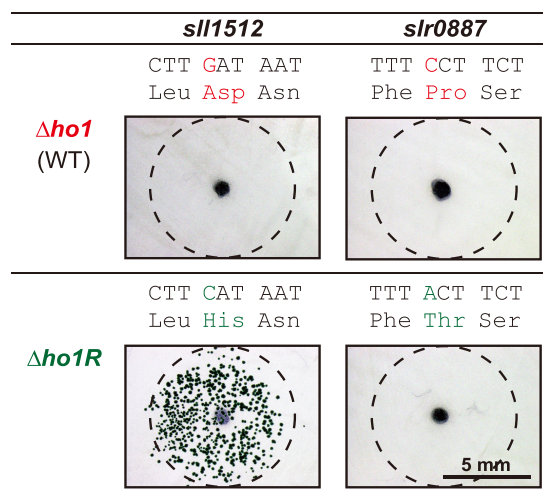


FIGURE 2. The transformation analysis to identify the mutation site important for $\Delta ho1R$. Left and right panels show the results of the transformation assay with DNA fragments containing $sll1512$ and $slr0887$, respectively. DNA fragments amplified from the genome of $\Delta ho1$ and $\Delta ho1R$ were used in the top and bottom panels, respectively. We confirmed that $\Delta ho1$ carries the wild-type copies of $sll1512$ and $slr0887$. The black dots at the center indicate the points where DNA fragments were spotted (100 ng), and the dashed circles indicate the area where the DNA solutions were spread. The sequences above the pictures show the mutation sites in the $\Delta ho1R$. Red and green characters correspond to wild-type and $\Delta ho1R$ sequences, respectively.

indicated that the amino acid sequence of Sll1512 shows significant similarity to those of a MarR family transcriptional regulator from a variety of prokaryotes. Among MarR family proteins with known functions HxlR from *Bacillus subtilis* showed the highest similarity score except for many cyanobacterial homologues (supplemental Fig. S3). *B. subtilis* HxlR activates the expression of the $hxlAB$ operon for formaldehyde fixation in response to the presence of formaldehyde (24).

To elucidate the physiological function of Sll1512 in *Synechocystis* 6803, we isolated a mutant, $\Delta sll1512$, that lacked the $sll1512$ gene (Fig. 3, A and B). $\Delta sll1512$ grew poorly under low-oxygen conditions, although it grew as vigorously as the wild type under aerobic conditions (Fig. 3, C and D). We determined the contents of PC and Chl (E and F). The PC content of the $\Delta sll1512$ cells was the same as that of the wild type under both aerobic and low-oxygen conditions (Fig. 3E). On the other hand, the Chl content of $\Delta sll1512$ grown under low-oxygen conditions was greatly decreased to about 10% of the wild type, whereas the Chl content of aerobically grown cells was the same as that of the wild type (Fig. 3F). These results indicated that Sll1512 plays an important role for growth under low-oxygen conditions. The significant growth retardation under low-oxygen conditions in $\Delta sll1512$ would be caused by the drastic decrease in Chl.

We examined the effects of the $sll1512$ -deficiency on the transcript levels of the $chlA_{II}$ - $ho2$ - $hemN$ operon (Fig. 3G). Interestingly, the transcripts of all the three genes in the operon were not detected at all in the $\Delta sll1512$ cells grown even under low-oxygen conditions. The transcript for $psbA1$ was detected in a low level in both wild-type and $\Delta sll1512$ cells grown aerobically. Although the transcript level was greatly increased in the wild-type cells grown under low-oxygen conditions, it was kept low in the $\Delta sll1512$ cells grown under low-oxygen condi-

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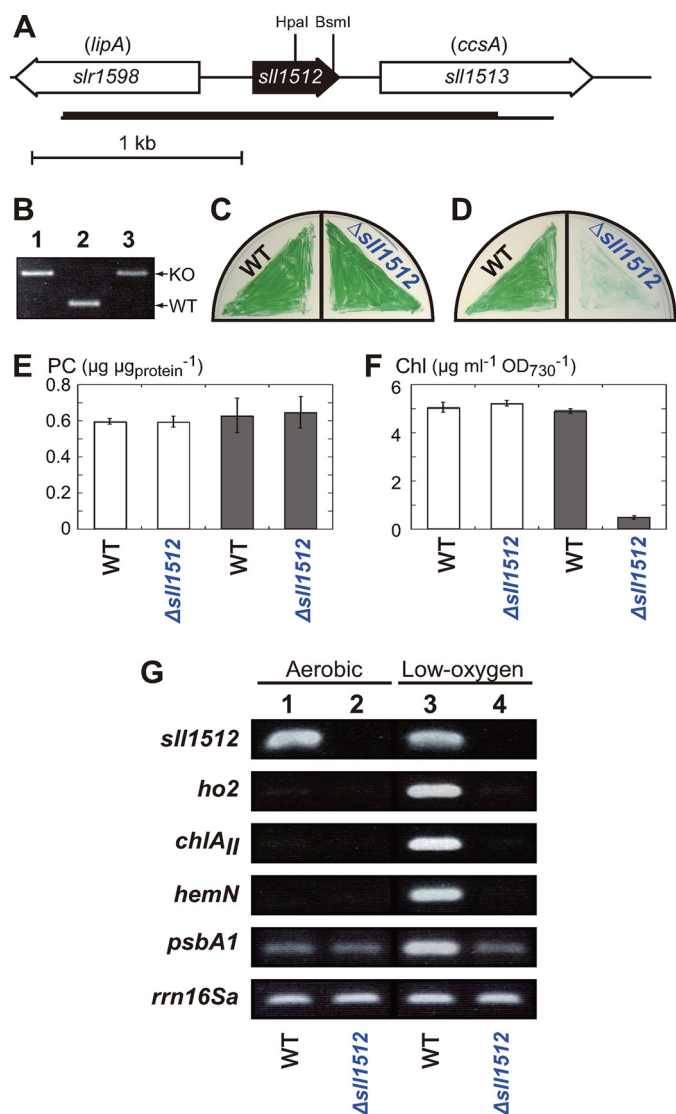


FIGURE 3. Characterization of Δ sll1512. A, gene arrangement of *sll1512* in the genome of *Synechocystis* 6803. The chromosomal region amplified by PCR to construct plasmids for targeted gene disruption is shown by a horizontal thin bar. The region between the HpaI and BsmI sites is replaced with a spectinomycin resistance cartridge in the Δ sll1512 mutant. B, colony PCR analysis to confirm the gene replacement in Δ sll1512. A chromosomal region (a horizontal thick bar in A) was amplified by PCR from single colonies on the selective agar plates (lane 3). Longer and shorter fragments correspond to the mutant (KO) and the WT copies of *sll1512*, respectively. As controls, the mutant (lane 1) and the wild-type (lane 2) fragments were amplified from the plasmid used for transformation and the wild-type genome, respectively. C and D, comparison of photoautotrophic growth of the wild-type and Δ sll1512. Cells were cultivated under aerobic (C) and low-oxygen (D) conditions for 5 days. E and F, comparison of PC (E) and Chl (F) contents in the wild-type and Δ sll1512. PC (E) and Chl (F) were extracted from cells grown photoautotrophically under aerobic (white bars) and low-oxygen (gray bars) conditions on the agar plates for 7 days. The data are mean \pm S.D. for three independent experiments. G, RT-PCR analysis for the transcript levels of *sll1512*, *ho2*, *chlA_{II}*, *hemN*, and *psbA1*. Total RNA was isolated from the wild-type (lanes 1 and 3) and Δ sll1512 (lanes 2 and 4) cells. Aerobic samples (lanes 1 and 2) were grown under low-oxygen conditions for 5 days and then transferred under aerobic conditions and grown for 2 days. Low-oxygen samples (lanes 3 and 4) were grown for 7 days under low-oxygen conditions. The housekeeping gene *rrn16Sa* was used as a control. Cycle numbers of RT-PCR for *sll1512* was 25, and those for the other genes are the same as in Fig. 1.

tions. These results suggested that Sll1512 is essential for the transcriptional activation of these genes under low-oxygen conditions. The transcript profile is in contrast to the constitu-

tive expression of these genes in the Δ ho1R. In Δ ho1R an Sll1512-D35H variant would behave as a constitutively active form resulting in the aberrant expression of the *chlA_{II}-ho2-hemN* operon and *psbA1* under aerobic conditions.

Gel Shift Assay of Sll1512 and the D35H Variant—To confirm the DNA binding ability of Sll1512, we performed a gel shift assay using two DNA fragments of the 312-bp *chlA_{II}* and 297-bp *psbA1* promoters and Sll1512 or the D35H variant (Fig. 4). The genes encoding Sll1512 and the D35H variant were overexpressed in *E. coli*, and the proteins (theoretical molecular mass, 16.4 kDa) were purified to almost homogeneity by affinity chromatography (supplemental Fig. S4A). There was no specific gel shift with the wild-type Sll1512 protein even at the highest concentration (700 nM) in either fragment. In contrast, an obvious mobility shift in both promoter fragments was detected in the assay with the D35H variant. Because all procedures, from crude extract preparation and protein purification to the assay, were carried out under normal aerobic conditions, the Sll1512 protein would be kept inactive without binding to the operator sequence. The binding features of Sll1512 and the D35H variant were consistent with our hypothesis (Fig. 5), in which the D35H variant is a constitutively active form to bind to the operator region under either aerobic or low-oxygen conditions.

DISCUSSION

The MarR family is one of the major transcriptional regulator families in prokaryotes and regulates a wide variety of cellular processes such as antibiotic resistance; virulence; oxidative stress; and environmental sensing of aromatic compounds, formaldehyde, and organic hydrogen peroxides (25, 26). In this work, we identified a MarR-family transcriptional regulator for the low-oxygen induction of genes for Chl and bilin biosynthesis in cyanobacteria for the first time. We propose to name it *sll1512* “chlR.”

Although cyanobacteria evolve oxygen by photosynthesis in the light like plants, they face hypoxia in a diurnal cycle because the oxygen level drops quickly after the onset of night (6). Many cyanobacteria also thrive in anoxic habitats such as hypolimnia, lake sediments, and soil (27). The transcriptional activator ChlR enables the cyanobacterium to produce Chl and bilin pigments even under low-oxygen environments. D1' encoded by *psbA1* is a special D1 isoform that is expressed only under low-oxygen conditions. In the field environment low-oxygen conditions are often associated with a high concentration of H₂S damaging to PSII. D1' is thought to help maintain PSII by rapid turnover of damaged conventional D1 isoforms under H₂S-rich conditions (23). Thus, it is reasonable that the *psbA1* is coregulated with the operon for Chl biosynthesis by ChlR.

MarR (multiple antibiotic resistance regulator) family regulatory proteins are small proteins consisting of 120- to 150-amino acid residues and form a homodimer to recognize specific palindromic nucleotide sequences upstream of the target genes (25). In most cases, MarR proteins function as repressors. The binding of a MarR regulatory protein on the operator region inhibits the transcription of the target genes by RNA polymerase, resulting in repression. Some MarR family members play a role as an activator. Such MarR members bind to a

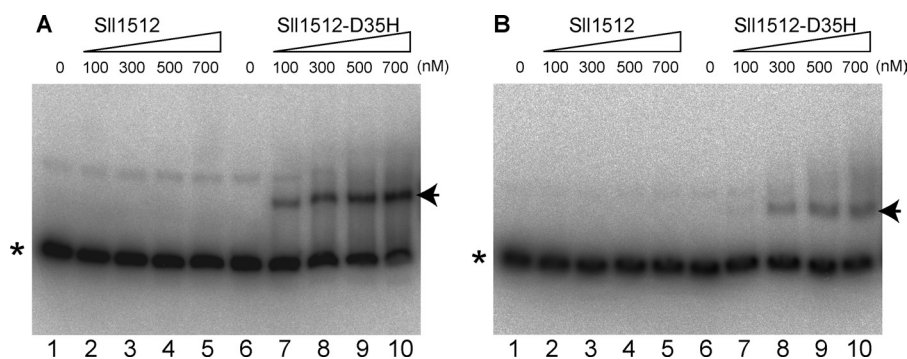


FIGURE 4. Gel mobility shift assays showing retardation of the ^{32}P -labeled DNA fragments of *chlA_{II}* (A) and *psbA1* (B) promoters by Sll1512 and Sll1512-D35H in polyacrylamide gel. Purified Sll1512 (lanes 1–5) and Sll1512-D35H (lanes 6–10) were added to the reaction mixtures to give the indicated concentrations of protein. Formed DNA-protein complexes are shown by the arrows, and asterisks indicate free probes.

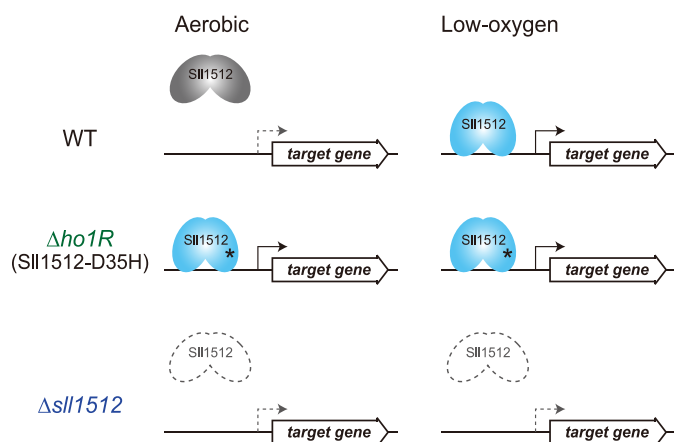


FIGURE 5. A working model of Sll1512 (ChlR) in *Synechocystis* 6803. In the wild-type cells, Sll1512 is maintained as an inactive form (gray) under aerobic conditions, so the target gene is not expressed (dotted gray arrows). When cells are exposed to low-oxygen conditions, Sll1512 is converted to an active form (light blue), and the target gene is transcribed (solid arrows). In the pseudorevertant $\Delta ho1R$, Sll1512 is fixed as an active form even under aerobic conditions because of the point mutation of D35H (asterisk), and the target gene is constitutively transcribed. In $\Delta sll1512$, there is no Sll1512 in the cell, and the target gene is not transcribed.

region close to the operator to activate the RNA polymerase to stimulate the transcription. We concluded that ChlR is an activator-type MarR member on the basis of the result of RT-PCR in $\Delta sll1512$ (Fig. 3G).

The *chlA_{II}-ho2-hemN* operon is conserved in *Synechococcus* 7002, and the expression is induced under low-oxygen conditions (15). Interestingly, a MarR-type transcriptional regulator (SYNPCC7002_A1993) is encoded just upstream of the operon in the opposite direction, which is a typical gene arrangement of MarR transcriptional regulator and the target genes. Because SYNPCC7002_A1993 showed about 67% identity to ChlR (supplemental Fig. S3), it may play a role as an activator of the operon in response to low-oxygen conditions like ChlR in *Synechocystis* 6803. We compared the upstream sequences of the *chlA_{II}* operon and the *psbA1* gene from *Synechocystis* 6803 and the *chlA_{II}* operon from *Synechococcus* 7002 to find a consensus motif that is recognized by ChlR. A palindromic sequence of TT(A/C)CC-N_{3/4}-GG(T/A)AA was found to be conserved commonly in the three regions (supplemental Fig. S5). This motif would be a binding site for ChlR. This consensus sequence is very similar to that (TTAC-N₅-GTAA) of CatR,

which is a repressor of the *catDE* operon encoding catechol 2,3-dioxygenase for quinone resistance in *B. subtilis* (28).

Crystal structures of many MarR family members have been reported in reduced, ligand-bound, and DNA-bound forms, starting with the crystal structure of MarR from *E. coli* (29), showing a common architecture with a domain for DNA binding called winged helix-turn-helix fold (25). Some MarR family members, including HxlR, constitute a group of regulatory proteins for oxidative stress responses. OhrRs in *B. subtilis* and *Xanthomonas campestris* have been most extensively studied as MarR members involved in oxidative stress sensing (30, 31). OhrR is a repressor of the gene *ohrA* encoding a thiol peroxidase that degrades organic hydroperoxides. They commonly possess oxidation-sensitive Cys residues in the N- and/or C-terminal region(s) by which they are divided into two subtypes, one-Cys-type and two-Cys-type (31). OhrR of *B. subtilis* represents a one-Cys-type regulator. Organic hydroperoxides react with a commonly conserved single Cys in N-terminal part, giving rise to Cys sulfenic acid (Cys-SOH) followed by rapid S-thiolation, resulting in dissociation from the operator to induce the *ohrA* gene expression (32). A representative of two-Cys-type is OhrR of *X. campestris* (31). The oxidation of a Cys (Cys-22) in the N-terminal part leads to the reaction with the other Cys in the C-terminal part (Cys-127') in the other protomer of the dimer, forming a disulfide bond (31). This S-S bond formation inactivates OhrR activity as a repressor by attenuation of the DNA binding ability.

ChlR appears to be a one-Cys-type regulator because only one Cys (Cys-25) of five Cys is conserved among all 12 cyanobacterial homologues (supplemental Fig. S3). In our hypothesis, ChlR may have a modified Cys like sulfenic acid or S-thiolated found in OhrR under aerobic conditions, and the modified ChlR would be activated by the conversion of the modified Cys to reduced Cys with free thiol upon exposure to low-oxygen environments. Because the wild-type ChlR did not bind to DNA (Fig. 4), the ChlR protein purified from *E. coli* would be an inactive form with the modified Cys residue. We performed a gel shift assay with the wild-type ChlR in the presence of dithiothreitol (10 mM) or sodium dithionite (10 mM). However, no detectable mobility shift was detected (supplemental Fig. S4B). How the inactive ChlR is converted to an active form that binds to DNA like the D35H variant remains unknown.

Transcriptional Regulator for Chlorophyll Biosynthesis

In the crystal structure of OhrR from *B. subtilis*, the active Cys-15 is located adjacent to two Tyr residues (Tyr-29' and Tyr-40') and Met-25' that are from the other protomer (33). In the sequence alignment, Asp-35 of ChlR corresponds to Met-25 of OhrR (supplemental Fig. S3), suggesting that Asp-35' is located in the vicinity of the probable redox active Cys-25 in ChlR. It is possible that the substitution of Asp-35 with His affects the conformation of ChlR to be kept as the active DNA-binding form even though Cys-25 is modified under aerobic conditions or that the Cys-25 modification itself is inhibited in ChlR-D35H, resulting in the constitutive active form.

$\Delta sll1512$ showed a severe growth defect and drastic decrease of Chl content under low-oxygen conditions (Fig. 3, D and F). In contrast, the individual mutants of *chlA_{IP}*, *ho2* and *hemN*, which we isolated previously, showed only slight growth retardation with no such significant decrease in the Chl contents (11, 12, 14). The poor growth phenotype of $\Delta sll1512$ would be caused by the defect of some other unidentified genes under low-oxygen conditions. Further study is needed to identify the whole set of the ChlR regulon.

Microarray analysis under low-oxygen conditions in *Synechocystis* 6803 has been reported (16, 23). Summerfield *et al.* (16) found that Hik31 histidine kinase is involved in negatively regulating gene expression of a large set of genes in response to low oxygen. Genes regulated by the Hik31-dependent system include subunits of photosystems I and II, phycobiliproteins, chaperons, and ATPase subunits. The other low-oxygen inducible genes, including *chlA_{IP}*, *ho2*, *hemN*, and *psbA1*, are regulated in a Hik31-independent system that has remained unknown until this work. Our results clearly demonstrated that ChlR is one of the key players in the Hik31-independent pathway. However, except for the four genes, there were a number of genes up-regulated by low-oxygen conditions such as *chlG*, *por*, *ctpA*, *hoxE*, and *flv4* (16). In our experiments, the up-regulation of these genes was not detected (supplemental Fig. S1). This discrepancy may be attributed to a difference in culture conditions. Although Summerfield *et al.* (16) observed the difference in transcript levels after a 1-h exposure to low-oxygen conditions of aerobically grown cells, we compared the transcript levels in cells grown under low-oxygen conditions (7 days) with those in cells after 2-day exposure to aerobic conditions. Our results, taken together with Summerfield *et al.* (16), suggest that the Hik31-independent regulatory system consists of more than two pathways, including the ChlR pathway. Further research is needed to elucidate the complex regulatory system to allow fine-tuning of the cellular metabolism in response to hypoxia.

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REFERENCES

1. Stal, L. J., and Zehr, J. P. (2008) in *The Cyanobacteria: Molecular Biology, Genomics and Evolution* (Herrero A., Flores E., eds) pp. 423–446, Caister Academic Press, Norfolk, UK
2. Bernroither, M., Zamocky, M., Paier, M., Furtmüller, P. G., Peschek, G. A., and Obinger, C. (2008) Heme-copper oxidases and their electron donors in cyanobacterial respiratory electron transport. *Chem. Biodivers.* **5**, 1927–1961
3. Keeley, J.F. (1988) Photosynthesis in Quillworts, or why are some aquatic plants similar to cacti? *Plants Today* **1**, 127–132
4. Jørgensen, B.B., Revsbech, N.P., Blackburn, T.H., and Cohen, Y. (1979) Diurnal cycle of oxygen and sulfide microgradients and microbial photosynthesis in a cyanobacterial mat sediment. *Appl. Environ. Microbiol.* **38**, 46–58
5. Stal, L. J. (1995) Physiological ecology of cyanobacteria in microbial mats and other communities. *New Phytol.* **131**, 1–32
6. Steunou, A.S., Bhaya, D., Bateson, M. M., Melendrez, M. C., Ward, D. M., Brecht, E., Peters, J. W., Kühl, M., and Grossman, A. (2006) *In situ* analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 2398–2403
7. Chew, A. G., and Bryant, D. A. (2007) Chlorophyll biosynthesis in bacteria. The origins of structural and functional diversity. *Annu. Rev. Microbiol.* **61**, 113–129
8. Masuda, T., and Fujita, Y. (2008) Regulation and evolution of chlorophyll metabolism. *Photochem. Photobiol. Sci.* **7**, 1131–1149
9. Mochizuki, N., Tanaka, R., Grimm, B., Masuda, T., Moulin, M., Smith, A. G., Tanaka, A., and Terry, M. J. (2010) The cell biology of tetrapyrroles. A life and death struggle. *Trends Plant Sci.* **15**, 488–498
10. Stenbaek, A., and Jensen, P. E. (2010) Redox regulation of chlorophyll biosynthesis. *Phytochemistry* **71**, 853–859
11. Goto, T., Aoki, R., Minamizaki, K., and Fujita, Y. (2010) Functional differentiation of two analogous coproporphyrinogen III oxidases for heme and chlorophyll biosynthesis pathways in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol.* **51**, 650–663
12. Aoki, R., Goto, T., and Fujita, Y. (2011) A heme oxygenase isoform is essential for aerobic growth in the cyanobacterium *Synechocystis* sp. PCC 6803. Modes of differential operation of two isoforms/enzymes to adapt to low oxygen environments in cyanobacteria. *Plant Cell Physiol.* **52**, 1744–1756
13. Yilmaz, M., Kang, I., and Beale, S. I. (2010) Heme oxygenase 2 of the cyanobacterium *Synechocystis* sp. PCC 6803 is induced under a microaerobic atmosphere and is required for microaerobic growth at high light intensity. *Photosynth. Res.* **103**, 47–59
14. Minamizaki, K., Mizoguchi, T., Goto, T., Tamiaki, H., and Fujita, Y. (2008) Identification of two homologous genes, *chlA_I* and *chlA_{IP}*, that are differentially involved in isocyclic ring formation of chlorophyll *a* in the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* **283**, 2684–2692
15. Ludwig, M., and Bryant, D. A. (2011) Transcription profiling of the model cyanobacterium *Synechococcus* sp. strain PCC 7002 by Next-Gen (SOLiD™) sequencing of cDNA. *Front. Microbiol.* **2**, 41
16. Summerfield, T. C., Nagarajan, S., and Sherman, L. (2011) Gene expression under low-oxygen conditions in the cyanobacterium *Synechocystis* sp. PCC 6803 demonstrates Hik31-dependent and -independent responses. *Microbiology* **157**, 301–312
17. Kanesaki, Y., Shiwa, Y., Tajima, N., Suzuki, M., Watanabe, S., Sato, N., Ikeuchi, M., and Yoshikawa, H. (2012) Identification of substrain-specific mutations by massively parallel whole-genome resequencing of *Synechocystis* sp. PCC 6803. *DNA Res.* **19**, 67–79
18. Tajima, N., Sato, S., Maruyama, F., Kaneko, T., Sasaki, N. V., Kurokawa, K., Ohta, H., Kanesaki, Y., Yoshikawa, H., Tabata, S., Ikeuchi, M., and Sato, N. (2011) Genomic structure of the cyanobacterium *Synechocystis* sp. PCC 6803 strain GT-S. *DNA Res.* **18**, 393–399
19. Yamazaki, S., Nomata, J., and Fujita, Y. (2006) Differential operation of dual protochlorophyllide reductases for chlorophyll biosynthesis in response to environmental oxygen levels in the cyanobacterium *Leptolyngbya boryana*. *Plant Physiol.* **142**, 911–922
20. Nomata, J., Mizoguchi, T., Tamiaki, H., and Fujita, Y. (2006) A second nitrogenase-like enzyme for bacteriochlorophyll biosynthesis. Reconstitution of chlorophyllide *a* reductase with purified X-protein (BchX) and YZ-protein (BchY-BchZ) from *Rhodobacter capsulatus*. *J. Biol. Chem.* **281**, 15021–15028
21. Yamamoto, H., Nomata, J., and Fujita, Y. (2008) Functional expression of

- nitrogenase-like protochlorophyllide reductase from *Rhodobacter capsulatus* in *Escherichia coli*. *Photochem. Photobiol. Sci.* **7**, 1238–1242
22. Nishimura, T., Takahashi, Y., Yamaguchi, O., Suzuki, H., Maeda, S., and Omata, T. (2008) Mechanism of low CO₂-induced activation of the *cmp* bicarbonate transporter operon by a LysR family protein in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. *Mol. Microbiol.* **68**, 98–109
 23. Summerfield, T. C., Toepel, J., and Sherman, L. A. (2008) Low-oxygen induction of normally cryptic *psbA* genes in cyanobacteria. *Biochemistry* **47**, 12939–12941
 24. Yurimoto, H., Hirai, R., Matsuno, N., Yasueda, H., Kato, N., and Sakai, Y. (2005) HxlR, a member of the DUF24 protein family, is a DNA-binding protein that acts as a positive regulator of the formaldehyde-inducible *hxlAB* operon in *Bacillus subtilis*. *Mol. Microbiol.* **57**, 511–519
 25. Perera, I. C., and Grove, A. (2010) Molecular mechanisms of ligand-mediated attenuation of DNA binding by MarR family transcriptional regulators. *J. Mol. Cell. Biol.* **2**, 243–254
 26. Ellison, D. W., and Miller, V. L. (2006) Regulation of virulence by members of the MarR/SlyA family. *Curr. Opin. Microbiol.* **9**, 153–159
 27. Stal, L., and Moezelaar, R. (1997) Fermentation in cyanobacteria. *FEMS Microbiol. Rev.* **21**, 179–211
 28. Chi, B. K., Kobayashi, K., Albrecht, D., Hecker, M., and Antelmann, H. (2010) The paralogous MarR/DUF24-family repressors YodB and CatR control expression of the catechol dioxygenase CatE in *Bacillus subtilis*. *J. Bacteriol.* **192**, 4571–4581
 29. Alekshun, M. N., Levy, S. B., Mealy, T. R., Seaton, B. A., and Head, J. F. (2001) The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. *Nat. Struct. Biol.* **8**, 710–714
 30. Fuangthong, M., and Helmann, J. D. (2002) The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 6690–6695
 31. Panmanee, W., Vattanaviboon, P., Poole, L. B., and Mongkolsuk, S. (2006) Novel organic hydroperoxide-sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress. *J. Bacteriol.* **188**, 1389–1395
 32. Lee, J. W., Soonsanga, S., and Helmann, J. D. (2007) A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8743–8748
 33. Hong, M., Fuangthong, M., Helmann, J. D., and Brennan, R. G. (2005) Structure of an OhrR-*ohrA* operator complex reveals the DNA binding mechanism of the MarR family. *Mol. Cell* **20**, 131–141