ChlR Protein of Synechococcus sp. PCC 7002 Is a Transcription Activator That Uses an Oxygen-sensitive [4Fe-4S] Cluster to Control Genes involved in Pigment Biosynthesis

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Synechococcus sp. PCC 7002 and many other cyanobacteria have two genes that encode key enzymes involved in chlorophyll a, biliverdin, and heme biosynthesis: acsfI-ho2-hemN-desF, and hemF-hemN. Under atmospheric O2 levels, AcsF1 synthesizes 3,8-divinyl protoporphyrin from Mg-protoporphyrin IX monomethyl ester, Ho1 oxidatively cleaves heme to form biliverdin, and HEM oxidizes coproporphyrinogen III to protoporphyrin IX. Under microoxic conditions, another set of genes directs the synthesis of alternative enzymes AcsFII, Ho2, and hemN. In Synechococcus sp. PCC 7002, open reading frame SynPCC7002_A1993 encodes a MarR family transcriptional regulator, which is located immediately upstream from the operon comprising acsfI Ho2, hemN, and desF (the latter encodes a putative fatty acid desaturase). Deletion and complementation analyses showed that this gene, denoted chlR, is a transcriptional activator that is essential for transcription of the acsfI-ho2-hemN-desF operon under microoxic conditions. Global transcriptome analyses showed that ChlR controls the expression of only these four genes. Co-expression of chlR with yfp reporter gene under the control of the acsfI promoter from Synechocystis sp. PCC 6803 in Escherichia coli demonstrated that no other cyanobacterium-specific components are required for proper functioning of this regulatory circuit. A combination of analytical methods and Mössbauer and EPR spectroscopies showed that reconstituted, recombinant ChlR forms homodimers that harbor one oxygen-sensitive [4Fe-4S] cluster. We conclude that ChlR is a transcriptional activator that uses a [4Fe-4S] cluster to sense O2 levels and thereby control the expression of the acsfI-ho2-hemN-desF operon.

Significance: ChlR is a simple regulatory element that could facilitate expression of O2-sensitive proteins.

Microorganisms acclimate continuously to changes in their physicochemical environments, including changes in nutrient availability, energy sources, salinity, pH, and temperature. Light is the energy source for phototrophic organisms; and thus, light wavelength and total irradiance are usually among the most important environmental factors for phototrophic organisms. Cyanobacteria perform oxygenic photosynthesis; and therefore, the supply of reducing equivalents generated by the photosynthetic apparatus increases with increasing irradiance, but the oxygen levels inside cells as well as in the immediate micro-environment can fluctuate significantly because of photosystem II activity and changes in respiratory oxygen uptake (1). Thus, cyanobacteria can rapidly acclimate to diurnal shifts in light availability, to rapidly changing irradiance levels throughout the day, and to the accompanying changes in oxygen levels that result. Adjustments can be made by several mechanisms, including changes in gene expression; protein maturation, assembly and stability; post-translational modifications of enzymes; and even substrate availability for O2-dependent enzymes.

For the model cyanobacterium Synechococcus sp. PCC 7002 (hereafter Synechococcus 7002), the impact of physicochemical parameters has been extensively studied using global systems biological approaches, including transcriptomics, proteomics, and metabolomics (2–6). Some changes in culture conditions, such as dark incubation, cause dramatic changes in the transcriptome (2), whereas other conditions, such as limitation for nutrients, cause more specific and limited short term responses (3, 4). Large changes in transcript levels for the acsfI-ho2-hemN-desF operon, which encodes key enzymes required for chlorophyll a, phycocyanobilin, heme, and lipid biosynthesis, were observed in response to changes in oxygen (2). A similar gene cluster comprising the open reading frames (ORFs) sll1874 (acsF1/chiA1), sll1875 (ho2), and sll1876 (hemN) occurs in Synechocystis sp. PCC 6803 (hereafter Synechocystis 6803), and transcript levels for these three genes also coordinate an increase under microoxic conditions (7).

The acsfI, ho2, and hemN genes encode important enzymes that are involved in pigment biosynthesis: Mg-protoporphyrin monomethyl ester oxidative ring cyclase, heme oxygenase, and...
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coproporphyrinogen III oxidase, respectively. Many cyanobacterial genomes encode two genes that produce alternative enzymes for these functions. For example, in *Synechocystis* 6803, there are two Mg-protoporphyrin monomethyl ester oxidative ring cyclases, AcsF$_{II}$/ChlA$_{II}$ (sll1214) and AcsF$_{II}$/ChlA$_{II}$ (sll1874), respectively. AcsF$_{II}$/ChlA$_{II}$ catalyzes the formation of the isocyclic ring of chlorophyll *a* (converting Mg-protoporphyrin IX monomethyl ester into 3,8-divinyl protochlorophyllide) under atmospheric oxygen levels, whereas under microoxic growth conditions, the same reaction is mainly performed by AcsF$_{II}$/ChlA$_{II}$ (7). Interestingly, both enzymes are monooxygenases and require molecular O$_2$ as a substrate. These enzymes are unrelated to Mg-protoporphyrin monomethyl ester oxidative ring cyclases of the BchE type, which are O$_2$-independent enzymes of the radical S-adenosylmethionine superfamily that use water as the oxygen donor for formation of the 13'-oxo group of (bacterio) chlorophylls and are widely distributed in anoxygenic phototrophs (8–11).

Cyanobacteria as well as rhodophyte, glaucoyte, and cryptomonad algae use phycobiliproteins as major antenna pigments for photosynthesis (12, 13). Because phycobiliproteins have numerous linear tetrapyrrole (phycobilin) chromophores, these organisms are critically dependent on heme oxygenase activity for the production of the bilin chromophores of the light-harvesting phycobiliproteins. *Synechocystis* 6803 has two heme oxygenases, Ho1 (sll1184) and Ho2 (sll1875), which can oxidatively cleave the heme macrocycle in a reaction that requires both O$_2$ and reducing equivalents and which produces carbon monoxide and biliverdin, the precursor of all linear tetrapyrrole pigments (14). Like Ho1 of *Synechocystis* 6803 and the heme oxygenases of other organisms, Ho2 also requires molecular oxygen for its catalytic activity (15). Under microoxic conditions and especially at high irradiance levels, Ho2 is the major enzyme cleaving heme to form biliverdin. Ho1 catalyzes the same reaction under atmospheric O$_2$ levels (16, 17).

The third gene of the cluster expressed under microoxic conditions, hemN (sll1876), encodes an oxygen-independent coproporphyrinogen III oxidase (HemN type), which belongs to the radical S-adenosylmethionine protein superfamily (18). It harbors an oxygen-sensitive [4Fe-4S] cluster and requires S-adenosylmethionine for catalysis (19, 20). Besides hemN, the *Synechocystis* 6803 genome includes a gene for an oxygen-dependent coproporphyrinogen III oxidase (HemF, sll1185), which is a monooxygenase with a binuclear iron center (21, 22). In *Synechocystis* 6803, hemN has been shown to be induced under microoxic conditions, but hemF is required for growth at atmospheric O$_2$ levels (23). The role of a second hemN-like gene (sll1917) in *Synechocystis* 6803 remains unclear (23).

A microarray study in *Synechocystis* 6803 showed that the *psbA1* gene, encoding an alternative D1 subunit of photosystem II, and the *acsF$_{II}$/ho2-hemN* operon were the only four genes for which transcript levels increased substantially under microoxic conditions (24). Recently, a MarR-type transcriptional activator (sll1512) that apparently controls the expression of these four genes was described (25).

While Aoki *et al.* (25) were studying the product of sll1512, we identified a similar transcriptional regulator, the product of ORF SYNPC7002_A1993 in *Synechococcus* 7002. In the studies presented here, we show that the product of this ORF, ChlR, is a transcription activator that controls the expression of a single operon encoding four genes in *Synechococcus* 7002. Additionally, we show that this transcription factor activates transcription from the *acsF$_{II}$/promoter of *Synechocystis* 6803 in the absence of any other cyanobacterium-specific factors in *Escherichia coli*. Finally, we show that this transcription activator harbors a single oxygen-sensitive [4Fe-4S] cluster per homodimer that acts as the O$_2$ sensory prosthetic group.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions—** *Synechococcus* sp. strain PCC7002 wild-type and mutant strains were maintained in liquid culture and on 1.5% agar plates in medium A supplemented with 1 mg of NaNO$_3$ ml$^{-1}$ (designated as medium A$^+$) as described previously (2, 26). Cultures of the SynPCC7002_A1993 (ChlR) overexpression strain (under control of an ammonia-repressible promoter) that were used for RNA extractions were grown in a HEPES-buffered A medium (25 mM HEPES, pH 8.0 replaced 8.3 mM Tris-HCl, pH 8.2) containing either 12 mM NaNO$_3$ or 10 mM NH$_4$Cl (4). Liquid cultures were grown in tubes containing medium (25 ml) at 38 °C with continuous irradiation with 250 μmol of photons m$^{-2}$ s$^{-1}$. The cultures were sparged with either 1% (v/v) CO$_2$ in air (standard conditions) or 1% (v/v) CO$_2$ in N$_2$ (microoxic conditions). The following antibiotic concentrations were added to the medium when appropriate: 50 μg ml$^{-1}$ for spectinomycin and/or 20 μg ml$^{-1}$ for gentamycin. Cultures for growth rate determination were cultivated without antibiotics, whereas cultures for RNA analyses were grown in the presence of the respective antibiotics. Cell growth was monitored by measuring the optical density at 730 nm (OD$_{730}$nm; 1.0 OD$_{730}$nm = 1.0 ± 0.2 × 10$^6$ cells ml$^{-1}$) with a Genesys 10 spectrophotometer (Thermo-Spectronic, Rochester, NY). Cultures for RNA analyses were inoculated at an OD$_{730}$nm between 0.05 and 0.1 from precultures that had been grown under the same conditions. When these cultures reached an OD$_{730}$nm of 0.7, three independently grown, replicate cultures were pooled. Cells derived from 25-ml aliquots of the cultures were rapidly centrifuged (5 min, 5000 × g, 4 °C), and the cell pellets were rapidly frozen in liquid nitrogen and stored at −80 °C until required.

**Inactivation of chlR and Construction of Expression Systems—** To inactivate the chlR (SynPCC7002_A1993) gene of *Synechococcus* 7002, ~1000-bp regions immediately upstream and downstream of chlR were amplified by PCR with primers 1 and 2 for the upstream sequence and primers 3 and 4 for the downstream sequence (see Table 1 for oligonucleotide sequences). Oligonucleotides 2 and 3 introduced EcoRV sites 2 bases downstream from the start codon and 6 bases upstream of the stop codon. The PCR products for the upstream and downstream flanking regions were digested with EcoRV, and the *aadA* gene conferring streptomycin and spectinomycin resistance was excised as a 1091-bp Eco53Kl fragment from plasmid pSR2A (plasmids used and constructed in this study are listed in Table 2). The flanking regions and the *aadA* cassette were purified after electrophoresis of DNA fragments on agarose gels. The fragments were mixed at a 3:1:3 ratio of the upstream flap to the antibiotic resistance cassette to the
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TABLE 1
Plasmids used in this study

| Plasmids | Relevant characteristics | Phenotype | Source or Ref. |
|----------|-------------------------|-----------|---------------|
| pSRA2    | Contains a 1.1-kb aadA streptomycin and spectinomycin resistance cassette | Ap<sup>a</sup> Sm<sup>a</sup> Sp<sup>a</sup> | Agilent Technologies, Inc. (Santa Clara, CA) |
| pBluescript II KS (+) | Cloning vector; polylinker | | |
| pRL409   | Contains a 1.2-kb cat chloramphenicol resistance cassette and a 1.5-kb ermC erythromycin resistance cassette | Ap<sup>a</sup> Cm<sup>Em</sup> | This study |
| pLM1     | pBluescript II KS (+) derivative harboring the cat cassette flanked by the 5' and 3' flanking regions of desF | Ap<sup>a</sup> Cm<sup>nm</sup> | This study |
| pLM2     | pBluescript II KS (+) derivative harboring the ermC cassette flanked by the 3' flanking regions of chlR and desF | Ap<sup>a</sup> Em<sup>Em</sup> | This study |
| pAQ1Ex-ntR | Contains recombination sites for pAQ1 of Synechococcus 7002, pMB1 ori; 5<sup>a</sup>-del-aro<sup>a</sup>-aadA between recombination sites | Ap<sup>a</sup> Sm<sup>r</sup> Sp<sup>r</sup> | This study |
| pAQ1pcpEx | Contains recombination sites for pAQ1 of Synechococcus 7002, pMB1 ori; P<sub>cpcBA</sub>-aro<sup>a</sup>-aadCI between recombination sites | Ap<sup>a</sup> Gm<sup>r</sup> | This study |
| pLM3     | Harbors recombination sites for pAQ1 of Synechococcus 7002, pMB1 ori; P<sub>aro<sup>a</sup>-aro</sub>-aadCI flanked by recombination sites for pAQ1 of Synechococcus 7002, pMB1 ori | Ap<sup>a</sup> Gm<sup>r</sup> | This study |
| pET-42b  | pMB1 ori, lacI, T7lac promoter, aphAII | Km<sup>r</sup> | Novagen, EMD Millipore |
| pLM5     | pET-42b derivative containing T7lac-chlR<sub>7002</sub>, pMB1 ori, lacI, kanR | Km<sup>r</sup> | This study |
| pLM6     | pLM3 derivative with P<sub>aczF<sup>II</sup></sub>-napL<sub>7002</sub>, aacCI between pAQ1 recombination sites; pMB1 ori | Ap<sup>a</sup> Gm<sup>r</sup> | This study |
| pCDFDuet<sup>TM-1</sup> | CloI3 ori, lacI, 2 × T7lac promoter, aadA | Sm<sup>a</sup> Sp<sup>a</sup> | Novagen, EMD Millipore |
| pLM7     | pCDFDuet<sup>TM-1</sup> derivative with P<sub>aczF<sup>II</sup></sub>-napL<sub>7002</sub>, aacCI between pAQ1 recombination sites | Sm<sup>a</sup> Sp<sup>a</sup> | This study |
| pAQ1Ex::P<sub>cpcBA</sub> yfp<sup>c</sup> | Contains recombination sites for pAQ1 of Synechococcus 7002, pMB1 ori; P<sub>cpcBA-aro<sup>a</sup>-aadCI</sub> between recombination sites | Ap<sup>a</sup> Gm<sup>r</sup> | This study |

* Ap<sup>a</sup>, ampicillin resistance; Cm<sup>Em</sup>, chloramphenicol resistance; Em<sup>Em</sup>, erythromycin resistance; Gm<sup>r</sup>, gentamicin resistance; Km<sup>r</sup>, kanamycin resistance; Sm<sup>a</sup>, streptomycin resistance; Sp<sup>r</sup>, spectinomycin resistance.

TABLE 2
Oligonucleotide primers used in this study

| Number | Sequence (relevant restriction sites underlined, 5’−3’) |
|--------|-------------------------------------------------------|
| 1      | AAACTCTAGATCTCGTCGTTAAAGTCAGCCG (XbaI site underlined) |
| 2      | TTATCATCCATCTGAGCCACACATATCTTCATTG (EcoRV site underlined) |
| 3      | CCGAGCATACCGTTAATTTTTGGAACATACC (EcoRV site underlined) |
| 4      | TTTCGGAGACCCGCTCTGTTGTTAGGACC (XbaI site underlined) |
| 5      | AAACGTGAGGCTACCTGTATTTTACT (XbaI site underlined) |
| 6      | ATCTTTATCTCCAGCTGTTATG (XbaI site underlined) |
| 7      | AAAAGCCTAACTTGCTTTGTAATCC (XbaI site underlined) |
| 8      | TTTCGGAGACCCGCTCTGTTGTTAGGACC (XbaI site underlined) |
| 9      | AAATCATGATCTCGACTCCACACCTTTAATG (NcoI site underlined) |
| 10     | TTTGGCTCTTTAAGCGCCACACGGATCTAC (BamHI site underlined) |
| 11     | TTTGGCTCGGGATCGGACCAACCTTTAATG (NcoI site underlined) |
| 12     | AAAAGGATCATCTGACCTGACTGCTGCTG (BamHI site underlined) |
| 13     | AAAAGGATCATCTGACCTGACTGCTGCTG (BamHI site underlined) |
| 14     | AAAAGGATCATCTGACCTGACTGCTGCTG (BamHI site underlined) |
| 15     | ATACACCGGTTTTAATTTTTTTTAC (NcoI site underlined) |

downstream flank and ligated with T4 DNA ligase. The ligation products were directly used to transform Synechococcus 7002 as described previously (27).

For inactivation of desF, the flanking regions were amplified by PCR using primers 5 and 6 (upstream) and primers 7 and 8 (downstream), respectively. The resulting PCR products were subsequently cloned as XbaI/EcoRV (upstream flanking region) and HindIII/XhoI fragments (downstream flanking region), respectively, into pBluescript II KS (+); finally, the cat gene conferring chloramphenicol resistance was cloned as a 1213-bp EcoRV/HindIII fragment from pRL409 into this plasmid, resulting in pLM1. For deletion of the entire region comprising chlR, acsF<sub>II</sub>, ho2, hemN, and desF, the flanking regions were amplified using primers 3 and 9 (3’ of chlR) and primers 7 and 8 (3’ of desF). The resulting PCR fragments were cloned as XbaI/EcoRV and HindIII/XhoI fragments, respectively, into pBluescript II KS (+), and ermC conferring erythromycin resistance was cloned as a 1503-bp EcoRV/HindIII fragment from pRL409 into the same plasmid, resulting in pLM2. XhoI-linearized pLM1 and Scal-linearized pLM2 were used to transform Synechococcus 7002.

To introduce the chlR gene into plasmid pAQ1 of Synechococcus 7002 under control of an inducible promoter, an expression vector having the P<sub>aro<sup>a</sup>ABC</sub> promoter of Synechocystis 6803 and aacCI gene conferring gentamycin resistance was constructed. A 478-bp EcoRI/NdeI fragment comprising the P<sub>aro<sup>a</sup>ABC</sub> promoter region of Synechocystis sp. PCC 6803 and a His tag sequence from pAQ1Ex-ntR (28) was cloned into pAQ1pcpEx that had had aacCI as a drug marker, resulting in pLM3. The chlR gene from Synechococcus 7002 was amplified using primers 10 and 11, and the resulting PCR product was cleaved with NcoI and BamHI, resulting in a 199-bp NcoI fragment and a 187-bp NcoI/BamHI fragment. The NcoI/BamHI fragment was first cloned into NcoI/BamHI-digested pLM3 followed by cloning the NcoI fragment into the NcoI-digested pLM3 derivative, finally resulting in pLM4. Both the orientation of the NcoI fragment and the whole PCR-amplified region were confirmed by sequencing. Through NcoI cloning, the His tag-coding sequence of pLM3 was removed, resulting in a non-tagged gene product. However, introduction of the NcoI site at the 5’-end of the chlR gene caused a threonine to
alanine exchange of the second amino acid in the resulting protein. The spectinomycin-resistant chlR deletion mutant strain was transformed with Scal-linearized pLM4.

A Strep-tagged variant of ChlR was constructed for expression and subsequent purification from E. coli. An N-terminal Strep tag-coding sequence and restriction sites were fused to the chlR coding sequence by PCR using primers 12 and 13, and the resulting PCR product was cleaved with Ndel and BamHI, resulting in a 422-bp Ndel/BamHI fragment. This fragment was cloned into NdeI/BamHI-digested pET-42b expression vector, yielding pLM5. A reporter construct based on the yellow fluorescent protein (YFP) was designed for testing expression levels from the acsF_H promoter sequence. To probe expression in Synechococcus 7002, the acsF_H promoter region of Synechocystis 6803 was amplified by PCR using primers 14 and 15. The PCR product was digested and cloned with EcoRI and NcoI, and the resulting 581-bp EcoRI/NcoI fragment was cloned into EcoRI/NcoI-digested pLM3, replacing the P_RNABCD promoter and yielding pLM6. Finally, Scal-linearized pLM6 was transformed into wild-type Synechococcus 7002. For expression experiments in E. coli, a 1515-bp EcoRI/XbaI fragment from pLM6, including the Synechocystis 6803 P_acsF_H sequence and yfp, was cloned into an EcoRI/XbaI fragment of pCDFDuet™-1, which only included the origin of replication and the adaA resistance marker, yielding pLM7. For expression experiments in E. coli, plasmids pLM5 and pLM7 were transformed either separately or together into E. coli BL21(DE3). All expression constructions for Synechococcus 7002 and E. coli were verified by DNA sequencing.

RNA Preparation, RNA Sequencing, and Data Analysis—RNA samples for subsequent cDNA library construction were prepared from frozen cell pellets derived from 25-ml aliquots of liquid culture (pooled from three independent cultures). The RNA preparation and quantitation were performed as described previously (2). Construction of cDNA libraries and SOLiD™ sequencing was performed in the Genomics Core Facility at The Pennsylvania State University (University Park, PA). The cDNA libraries were constructed using the SOLiD Whole Transcriptome Analysis kit (Applied Biosystems) and were barcoded by using the SOLiD Transcriptome Multiplexing kit (Applied Biosystems). The SOLiD ePCR kit and SOLiD Bead Enrichment kit (both Applied Biosystems) were used for processing the samples for sequencing, and the SOLiD 5500 protocol (Applied Biosystems) was used for sequencing. The sequence data have been submitted to the NCBI Sequence Read Archive (SRA) under accession numbers SRX275952 to SRX275958 and SRX268824.

Mapping of cDNA sequences was performed by using the Burrows-Wheeler algorithm (29). The Synechococcus 7002 genome and the sequences for the various drug marker cassettes used were used as the reference genome; four mismatches within the 50-bp reads were allowed (>90% sequence identity). Sequences mapping to ribosomal RNA-coding regions and reads that did not map uniquely were disregarded. The methods for counting the sequences covering each ORF, for calculating the relative transcript abundance (RTA)^3 for each ORF, and for comparing RTAs between different data sets were performed as described previously (2). The probability (p values) for equal RTA in the respective comparisons was calculated for each ORF either by using the z-test or the χ² test as appropriate (for more details, see Ref. 2). The data for all protein-coding ORFs derived from these analyses are listed in supplemental Tables S1 and S2.

Overexpression and Purification of N-terminally Strep-tagged ChlR—For production of N-terminally Strep-tagged ChlR (N_strepChlR), E. coli cells were cultivated in 1 liter of Luria-Bertani medium (10 g liter⁻¹ tryptone, 5 g liter⁻¹ yeast extract, 10 g liter⁻¹ NaCl) containing 30 µg ml⁻¹ kanamycin in 2.8-liter flat bottom flasks at 37 °C with shaking at 120 rpm. When the cultures reached an OD₆₀₀ nm of 0.6, chlR expression was induced by adding 0.1 mM isopropyl 1-thio-β-D-galactopyranoside, and the culture was further incubated for 4 h. Cells were harvested by centrifugation (10 min, 5000 × g, 4 °C), washed in Buffer A (100 mM Tris/HCl buffer at pH 8.0, 150 mM NaCl), frozen in liquid nitrogen, and stored at −80 °C until required.

Purification of N_strepChlR was performed under oxic conditions. Cells were resuspended in Buffer A at a ratio of 8 ml of buffer/1 g of cells (wet weight), and lysozyme was added to a final concentration of 1 mg ml⁻¹. The cell suspension was stirred at room temperature for 20 min, and cells were disrupted by sonication (Branson Sonifier 450, three intervals at 30 s at level 2.5 and 70%). The sample was kept on ice during sonication. The cell extract was cleared by ultracentrifugation (100,000 × g, 60 min, 4 °C). The resulting soluble extract was loaded on a column containing Strep-Tactin Superflow resin (Iba, Göttingen, Germany) (5-mL bed volume equilibrated with Buffer A). The column was washed with 10 column volumes of Buffer A and eluted with 5 × 3 ml of elution buffer (Buffer A containing 5 mM D-desthiobiotin). Fractions were collected, and protein-containing fractions were pooled and concentrated using a centrifugal ultrafiltration device (Amicon Ultra centrifugal filter, Ultracel 10,000, EMD Millipore, Billerica, MA; 5000 × g). The sample was washed twice using Buffer A and concentrated again to dilute the D-desthiobiotin. The protein concentration was determined by the Bradford method (30) using bovine serum albumin as a standard. The purity of the samples was estimated by visual inspection of SDS-polyacrylamide gels stained by Coomassie Brilliant Blue G-250 (31). Quantitative amino acid analysis of N_strepChlR samples was performed at the Molecular Structure Facility at the University of California-Davis.

Fe-S Cluster Reconstitution and Chemical Determination of Iron—Reconstitution of Fe-S clusters was performed under anoxic conditions as described previously (32). In brief, dithiothreitol (10 mM final concentration), FeCl₃ (0.5 mM final concentration), and Na₂S (0.8 mM final concentration) were added stepwise to a solution of N_strepChlR (at a final concentration of 0.1 mM in Buffer A). After reconstitution, the sample was concentrated by ultrafiltration (Amicon Pressure Cell, EMD Millipore) with a 10,000-molecular weight cutoff membrane and

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3 The abbreviations used are: RTA, relative transcript abundance; N_strepChlR, N-terminally Strep-tagged ChlR; T, tethals; mT, miltiletas.
subjected to ion-exchange chromatography. Molecular sieve chromatography of N\textsubscript{Strep}-ChlR was carried out according to a procedure described previously (33) using an AKTA (GE Healthcare) liquid chromatography system in a Coy anaerobic chamber. The sample was purified on a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) equilibrated with Buffer A at a flow rate of 0.5 ml min\textsuperscript{-1}. This column was previously calibrated with molecular weight markers as described (34). Protein-containing fractions were pooled and concentrated using a centrifugal ultrafiltration device (Amicon Ultra centrifugal filter, Ultracel 10,000, EMD Millipore). Samples prepared for Mössbauer spectroscopy were reconstituted using \textsuperscript{57}Fe by the same procedure. Chemical iron determination was performed as described previously (35, 36).

UV-Visible Spectroscopy, Low Temperature EPR, and Mössbauer Spectroscopy—UV-visible spectra were recorded using a modified Cary 14 spectrophotometer (On-Line Instrument Systems, Bogart, GA). Diluted, reconstituted N\textsubscript{Strep}-ChlR samples were aliquoted into air-tight quartz cuvettes under anoxic conditions. After spectra of the anoxic samples were recorded, the cuvettes were opened to expose the sample to oxygen. After mixing the sample with air and incubating the sample for 20 min at room temperature, spectra of the oxygen-exposed samples were recorded.

N\textsubscript{Strep}-ChlR (300 \mu l, \textsuperscript{57}Fe-labeled) that had been reconstituted anoxically for Mössbauer spectroscopy was aliquoted into Mössbauer cups under anoxic conditions, frozen, and stored in liquid nitrogen until measured. A sample of O\textsubscript{2}-exposed reconstituted N\textsubscript{Strep}-ChlR was prepared by exposing the above sample to air, pipetting to mix the solution several times, and then incubating it at ambient atmosphere on ice for 15 min before freezing the sample in liquid nitrogen. Samples for EPR spectroscopy were prepared by diluting the samples 10-fold with Buffer A, placed in calibrated EPR tubes (Wilmad Lab Glass, Vineland, NJ), and frozen anoxically by slow immersion of the tube in liquid nitrogen. EPR samples of reduced N\textsubscript{Strep}-ChlR were prepared by addition of 9 \mu l of 100 mM sodium dithionite in 1 \textmu l of HEPES buffer, pH 7.5, to a 300-\mu l sample. After adding dithionite solution, the sample was mixed, incubated for a few minutes on ice, and then frozen as described above.

EPR spectra were acquired on a Bruker ESP300 CW X-Band spectrometer (operating at approximately 9.48 GHz) equipped with a rectangular cavity (TE102) and a continuous flow cryostat (Oxford 910) with a temperature controller (Oxford ITC 503). Spin quantitation was carried out relative to a Cu\textsuperscript{2+-EDTA standard.

Mössbauer spectra were recorded on spectrometers from WEB Research (Edina, MN). The spectrometer used to acquire the weak field spectra was equipped with a Janis SVT-400 variable temperature cryostat. The spectrometer used to acquire the strong field spectra was equipped with a Janis 8TMOSSEDM-125VT variable temperature cryostat. The external magnetic field was applied parallel to the y beam. All isomer shifts quoted are relative to the centroid of the spectrum of \textalpha-iron metal at room temperature. Simulation of the Mössbauer spectra was carried out by using the WMOSS spectral analysis software (WEB Research, Edina, MN).

\textbf{RESULTS}

\textit{Deletion of chlR Affects Growth at Lower O\textsubscript{2} Levels}—To investigate the function of ORF SynPCC7002_A1993, which was annotated as a putative transcriptional regulator, a deletion mutant was constructed by completely replacing the ORF by a drug resistance cassette (aadA) (Fig. 1A). After transformation, the wild-type and mutant alleles segregated completely (Fig. 1B), which indicated that \textit{chlR} is not an essential gene under the conditions used for selection of transformants and segregation of alleles. Growth of the \textit{chlR} mutant strain was indistinguishable from that of the wild type when agar plates were incubated in air (Fig. 2A). However, when plates were incubated under an
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**Deletion of chlR Abolishes O₂-dependent Regulation of the Low O₂-induced Operon**—In a previous study on the effects of light, darkness and oxygen on the transcriptome of *Synechococcus* 7002, we showed that transcript levels of an operon comprising *acsF*₂, *ho2*, *hemN*, and *desF* clearly responded to changing O₂ levels. Compared with standard conditions, the relative transcript abundance for these four genes increased when cells were grown under microoxic conditions (2) (see Fig. 3A). Besides these four genes, a few other genes showed increased mRNA levels under microoxic conditions, notably *isiA* and *isiB* (Table 3). Global transcriptomes for the *chlR* mutant strain obtained for cultures grown under the same conditions (microoxic versus standard) were essentially identical (Fig. 3B). Transcript levels for *acsF*₂-*ho2-* *hemN*, and *desF* in cells grown under these two conditions did not differ significantly (p values >0.1; Table 3). The transcriptomes of the *chlR* mutant and the wild type were identical when cultures were grown under standard conditions (Fig. 3C). However, when the two strains were grown under microoxic conditions, the transcript levels for *acsF*₂-*ho2-* *hemN*, and *desF* were much lower in the *chlR* mutant compared with the wild type (Fig. 3D and Table 3).

**Controlled Expression of chlR Restores Expression of the acsF*₂-*ho2-* *hemN*-desF Operon**—For the expression of ChlR in the *chlR* deletion mutant strain, the gene was reinserted by taking advantage of the previously described expression system (37) that inserts a gene of interest into pAQ1, which is the smallest plasmid of *Synechococcus* 7002 and which has the highest copy number of the six plasmids in this strain (37). For expression experiments, the controllable *nrtABCD* promoter of *Synechocystis* 6803 that is induced in the absence of ammonia (39) was selected to be able to control the expression levels of *chlR*. Fig. 3E compares the transcriptomes obtained for permissive conditions for *chlR* expression (i.e., cells were grown with nitrate as the nitrogen source) with the transcription data obtained for the *chlR* mutant when both cultures were grown with nitrate under microoxic conditions. Transcript levels for *acsF*₂-*ho2-* *hemN*, and *desF* were 90–250-fold higher in the cells expressing *chlR* compared with the “background” levels for these transcripts observed in the *chlR* deletion mutant (Table 3). The much higher transcript levels for the *acsF*₂-*ho2-* *hemN*-desF operon in the complemented strain clearly showed that the transcriptional regulation of these genes had been fully restored (Fig. 3E). Notably, transcript levels for *isiA* and *isiB*, which were increased in the wild-type strain under microoxic conditions, did not respond to complementation (Table 3).

A comparison of the transcriptome obtained for cells of the complemented *chlR* expression strain that were grown under microoxic conditions with that of cells grown at an atmospheric...
O$_2$-sensitive Transcription Activator in Synechococcus 7002

A

B

C

D

E

F
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### Table 3

| Gene name | WT microoxic/ std | $\Delta$chIR microoxic/ std | $\Delta$chIR/WT std | $\Delta$chIR/WT microoxic | $\Delta$chlR Comp NO$_3$/NH$_4^+$ microoxic/std | $\Delta$chlR Comp NO$_3$/NH$_4^+$ microoxic/std | $\Delta$chlR Comp NH$_4^+$ microoxic/std | $\Delta$chlR Comp NO$_3$/WT microoxic | $\Delta$chlR Comp NO$_3$/WT microoxic |
|-----------|------------------|-----------------------------|--------------------|--------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|------------------------------------------|------------------------------------------|
| chlR      | 1.56$_b^a$       | ND$_b^a$                    | 0.00               | 0.13                     | 26.84                                       | 8.37                                        | 0.30                                        | 0.95$_b^a$                               | 85.01                                   | 10.64                                    |
| acsF$_H$  | 36.82            | 2.05$_b$                   | 0.77$_b$          | 0.04                     | 4.14                                        | 155.44                                      | 40.89                                       | 1.09$_b$                                 | 87.74                                   | 3.75                                     |
| ho2       | 24.95            | 0.46$_b$                   | 0.90$_b$         | 0.03                     | 6.03                                        | 120.67                                      | 24.38                                       | 1.22$_b$                                 | 245.20                                  | 4.04                                     |
| hemN      | 31.86            | 1.82$_b$                   | 0.87$_b$         | 0.05                     | 6.64                                        | 225.74                                      | 51.82                                       | 1.52$_b$                                 | 114.67                                  | 5.69                                     |
| desF      | 36.86            | 0.61$_b$                   | 3.42$_b$         | 0.07                     | 1.85$_b$                                   | 82.89                                       | 40.96                                       | 0.91$_b$                                 | 126.32                                  | 8.51                                     |
| isiA      | 8.88             | 0.74$_b$                   | 1.36$_b$         | 0.12                     | 0.25                                        | 0.50                                        | 1.79                                        | 0.91$_b$                                 | 1.59$_b$                                | 0.19                                     |
| isiB      | 11.93            | 5.47$_b$                   | 0.51$_b$         | 0.24                     | 0.40                                        | 0.47                                        | 0.99$_b$                                   | 0.85$_b$                                 | 0.80$_b$                                 | 0.19                                     |

$^a$ p value $>0.1$.

O$_2$ level with both cultures grown with nitrate as nitrogen source showed that transcript levels for $acsF_H$, ho2, hemN, and desF were much higher under microoxic conditions, whereas isiA and isiB levels were essentially unchanged (Fig. 3F). This result confirms that regulation of only the $acsF_H$-ho2-hemN-desF operon is highly O$_2$-dependent. When the chlIR expression strain was grown with ammonia as the nitrogen source (i.e. chlIR expression is repressed), the transcriptomes of cultures grown under microoxic and standard oxygen conditions were nearly identical (Fig. 4A). When the transcriptome of cells of the chlIR expression strain that were grown with ammonia as nitrogen source was compared with the transcriptomes of cultures grown with nitrate as nitrogen source that harbored the plasmid pAQ1::$acsF_H$-ho2-hemN-desF operon under atmospheric oxygen conditions. The YFP content in cells of Synechococcus 7002 was monitored as YFP-derived fluorescence at 526 nm. In cultures harboring pAQ1::$acsF_H$-ho2-hemN-desF::yfp, relatively strong YFP fluorescence was observed when the cultures were grown under microoxic conditions (Fig. 5). This result clearly shows that ChlR of Synechococcus 7002 recognized the Synechocystis 6803 promoter. The signal intensity reached about 25% of that observed for the positive control having yfp under the control of the strong cpcBA promoter. The signal intensity of the ChlR-YFP fusion was about 6-fold lower than that for cells grown under microoxic conditions, which is consistent with the much lower transcript levels observed for the $acsF_H$-ho2-hemN-desF operon under atmospheric oxygen conditions.

Interaction of ChlR with $P_{acsF-H}$ is Independent of Other Cyanobacterium-specific Elements: After demonstrating that ChlR could interact with and regulate the $acsF_H$ promoter from Synechocystis 6803, we investigated whether this interaction relies on other cyanobacterium-specific elements. Therefore, chlIR (as an N-terminally Strep-tagged derivative) and yfp (under control of $P_{acsF-H}$) were expressed from separate plasmids (see “Experimental Procedures”) in E. coli BL21(DE3). Maturation of the chromophore of YFP does not occur in the absence of O$_2$ (41). Conveniently, the conditions to allow maturation of the YFP chromophore are incompatible with transcriptional activation, so these two processes can be temporally separated. Chromophore maturation was incomplete in E. coli cultures that were grown under the nearly anaerobic conditions used. To achieve full YFP fluorescence signals in E. coli cells, a prolonged incubation (about 2 h) of the cells in a buffer at room temperature and in the presence of O$_2$ was required (data not shown).

$E. coli$ strains with only one of these two components (either chlIR or $P_{acsF-H}$::yfp) did not show any YFP-derived fluorescence, and only the strains carrying both plasmids allowed YFP.

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**Figure 3. Changes in the RTA of wild-type Synechococcus 7002, ΔchlIR mutant, and this mutant complemented with chlIR under standard or microoxic conditions.** The scatter plots show the RTAs of transcripts from wild-type cells grown under microoxic conditions compared with cells grown under atmospheric O$_2$ (A), the RTAs for cells of the ΔchlIR mutant grown under microoxic conditions compared with those for cells grown under atmospheric O$_2$ (B), C, scatter plot showing the RTAs of cells of the ΔchlIR mutant compared with those for the wild type when both strains were cultivated under atmospheric O$_2$, D, scatter plot showing the RTAs for cells of the ΔchlIR mutant compared with those of the wild type when cultures were grown under microoxic conditions, E, scatter plot showing the RTAs of the cells for the complemented ΔchlIR mutant under derepression conditions (using NO$_3^-$ as the nitrogen source) to allow expression of chlIR compared with the RTA values for the ΔchlIR mutant (both strains were grown under microoxic conditions). F, scatter plot showing a comparison of the complemented ΔchlIR mutant under derepression conditions (using NO$_3^-$ as the nitrogen source) to allow expression of chlIR compared with the RTA values for the ΔchlIR mutant (both strains were grown under microoxic conditions).
expression in *E. coli* (data not shown). The YFP-derived fluorescence signal was intense in cultures grown under microoxic conditions. The maximal fluorescence intensity was about half of that obtained with the very strong *cpcBA* promoter, which also supports YFP expression in *E. coli*, for equal cell numbers (Fig. 6). Similar to the situation in *Synechococcus* 7002, when *E. coli* cells were grown under oxic conditions with strong aeration, the fluorescence signals were 4–5-fold lower compared with cells grown under microoxic conditions. These results indicate that YFP expression controlled by ChlR relies on the O$_2$ level and interaction between the transcription activator and the promoter. No other components specific to cyanobacteria are apparently required.

**ChlR Harbors an Oxygen-labile Fe-S Cofactor**—N-terminally Strep-tagged ChlR was heterologously overproduced in *E. coli* BL21(DE3) and purified to homogeneity under oxic conditions by Strep-Tactin affinity chromatography (Fig. 7A). Preliminary data showed that heterologously produced NStrepChlR contained substoichiometric amounts of iron, and UV-visible spectroscopy revealed a faint absorbance that suggested the presence of an Fe-S cofactor. The small Fe-S cluster yield suggested either that the Fe-S cluster biosynthesis machinery of the host cells could not provide sufficient Fe-S clusters to allow for complete maturation of ChlR or that the purification under oxic conditions led to its complete degradation. By enhancing the iron supply during cultivation and by conducting the purification of NStrepChlR under anoxic conditions, the iron content and the characteristic UV-visible absorbance could be increased to some extent, but ChlR was still mostly devoid of Fe-S clusters.

**FIGURE 4.** Changes in the RTA of the *Synechococcus* 7002 ∆chlR deletion mutant complemented with *chlR* under different O$_2$ levels and on different nitrogen sources. The scatter plots show the RTA values for cells of the ∆chlR mutant complemented with *chlR* when cells were grown under microoxic conditions relative to atmospheric O$_2$ level (cells were grown with ammonia as the nitrogen source) (A), the RTA values for cells of the ∆chlR mutant complemented with *chlR* that had been grown under standard conditions with nitrate or ammonia as the nitrogen source (B), and the RTA values for cells of the ∆chlR mutant complemented with *chlR* when grown under microoxic conditions with nitrate or ammonia as the nitrogen source (C). The gray lines indicate 2-fold changes in transcript level. Selected genes are identified by name or abbreviated locus tag number. *Comp, chlR*-complemented strain.
To determine the nature of the metal-containing cofactor, N$_{\text{Strep}}$ChIR was next purified under oxic conditions from cells grown oxicly, and the protein was then chemically reconstituted under anoxic conditions. After repurification of the protein from the reconstitution solution, the protein had a dark brownish color, and a broad absorption band between 370 and 440 nm was observed in the UV-visible spectrum immediately after reconstitution under an O$_2$-free atmosphere (Fig. 7B). This absorption spectrum is typical of the S$\rightarrow$Fe charge transfer bands characteristic of Fe-S proteins. Quantitative amino acid analysis showed that the protein concentration values obtained by the Bradford assay overestimated the protein concentration and that a correction factor of 0.54 was required. An iron analysis for reconstituted N$_{\text{Strep}}$ChIR revealed the presence of 4.06$\pm$0.07 iron atoms per N$_{\text{Strep}}$ChIR homodimer (see below).

When solutions containing reconstituted N$_{\text{Strep}}$ChIR were exposed to oxygen (by opening the cuvette, mixing the protein solution with air, and incubating at room temperature for 20 min), the absorbance in the 370–440-nm region of the UV-visible spectrum decreased dramatically (Fig. 7B). Subsequent removal of oxygen and reduction of the same sample with 1 mM sodium dithionite did not restore the original signal but led to a further decrease of the absorbance. These data suggest that the Fe-S cluster in N$_{\text{Strep}}$ChIR is irreversibly modified by oxygen.
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ChlR Is a Homodimer That Binds One [4Fe-4S] Cluster per Homodimer—To determine the stoichiometry and type of Fe-S cofactors in ChlR, we used a combination of analytical and spectroscopic methods (42). Recombinant apo-NStrepChlR was reconstituted under anoxic conditions, and the resulting protein was chromatographed on a Sephacryl S-200 column to remove excess Fe-S reconstitution reagents and to determine the molecular weight of the protein (Fig. 8). By comparison of the elution time with those for proteins of known molecular weight, the molecular weight of NStrepChlR was determined to be ~33,000. Considering that the calculated mass of an NStrep-ChlR monomer is 15,732 Da, this result establishes that reconstituted NStrepChlR is a homodimer in solution.

The 4.2-K/53-mT Mössbauer spectrum of NStrepChlR reconstituted with ⁵⁷Fe under anoxic conditions (Fig. 9A, top, vertical bars) is dominated by a quadrupole doublet with parameters (isomer shift δ = 0.45 mm/s and quadrupole splitting ΔE₀ = 1.09 mm/s, 74% of signal intensity; blue line) characteristic of [4Fe-4S]²⁺ clusters (43, 44). In addition, the pronounced shoulder at ~0.5 mm/s (green arrow) suggests the presence of a small amount of [2Fe-2S]²⁺ clusters (quadrupole doublet with δ = 0.28 mm/s and ΔE₀ = 0.54 mm/s, ~15% of signal intensity; green line). The remaining ~10% of the absorption is broad and featureless (see black arrows) and could emanate either from Fe-S clusters with half-integer spin ground state or from unspecifically bound iron. The EPR spectrum of NStrepChlR reconstituted under anoxic conditions (Fig. 9B, red line) demonstrated largely the absence of Fe-S clusters with half-integer spin ground state. The weak signal with gav = 2.006 could emanate from a small amount (~0.01 eq) of [3Fe-4S]⁺ cluster, but this small amount is beyond the detection limit of Mössbauer spectroscopy. Therefore, the broad features in the Mössbauer spectrum are assigned to unspecifically bound iron. The 4.2-K/6-T Mössbauer spectrum (Fig. 9A, bottom) confirms the presence of [4Fe-4S]²⁺ and [2Fe-2S]²⁺ clusters, which have diamagnetic ground states. The 6-T spectrum can be simulated with the parameters obtained from the 53-mT spectrum: asymmetry parameters η = 0.8 ([4Fe-4S]²⁺ cluster; blue line) and η = 0 ([2Fe-2S]²⁺ cluster; green line), respectively, and assuming an S = 0 ground state for both. Together with the iron and protein analyses, these results suggest the presence of 0.74 eq of [4Fe-4S]²⁺ clusters and 0.30 eq of [2Fe-2S]²⁺ clusters per NStrepChlR dimer.

The EPR spectrum of dithionite-reduced, reconstituted NStrepChlR (Fig. 9B, blue line) exhibits a nearly axial spectrum.

FIGURE 8. Elution profile for gel exclusion chromatography of reconstituted NStrep-ChlR on Sephacryl S-200 under anoxic conditions. The black line indicates absorption (relative units) at 400 nm, and the gray line indicates absorption (relative units) at 280 nm. The reconstituted NStrep-ChlR harboring an Fe-S cluster eluted at 65 ml and excess iron, polysulfides, and protein absorption (relative units) at 280 nm. The reconstituted NStrepChlR harboring indicates absorption (relative units) at 400 nm, and the line.

FIGURE 9. Mössbauer and EPR spectra of reconstituted NStrep-ChlR transcription activator. A, Mössbauer spectra of ⁵⁷Fe-labeled reconstituted NStrep-ChlR (630 μm homodimeric NStrep-ChlR). The spectra were recorded at 4.2 K in magnetic fields of 53 mT (top, vertical bars) and 6 T (bottom, vertical bars) applied parallel to the y beam. Simulations with parameters quoted in the text corresponding to the [4Fe-4S]²⁺ and [2Fe-2S]²⁺ clusters are shown in blue and green, respectively, whereas the black line corresponds to their summation. The green and black arrows indicate typical features associated with [2Fe-2S]²⁺ and adventitiously bound iron. B, continuous wave EPR spectra of anoxically reconstituted NStrep-ChlR (red line), anoxically reconstituted NStrep-ChlR after reduction with 5 mM sodium dithionite (DT) (blue line), and anoxically reconstituted NStrep-ChlR exposed to O₂ for 30 min (black trace). Experimental conditions were as follows: microwave power, 0.63 milliwatt; modulation amplitude, 0.5 mT; conversion time, 0.082 s; temperature, 10 K; microwave frequency, 9.480 GHz; sample concentration, 63 μm dimeric NStrep-ChlR.
with principal $g$ values of $g_x = 2.04, g_y = 1.92,$ and $g_z = 1.89$ and relaxation properties characteristic of those of $[4Fe-4S]^{2+}$ clusters (45). Spin quantitation using a Cu$^{2+}$ standard yielded 0.77 spin per NStrepChlR homodimer, suggesting that the $[4Fe-4S]^{2+}$ cluster is (nearly) quantitatively reduced.

Exposure of NStrepChlR reconstituted anoxically to O$_2$ was monitored by Mössbauer and EPR spectroscopies. The Mössbauer spectra recorded before and after O$_2$ exposure (Fig. 10, top and middle, respectively) reveal pronounced changes. The progress of the reaction can be demonstrated from the difference spectrum (Fig. 10, bottom) in which the features pointing downward are converted to those pointing upward upon O$_2$ exposure. Analysis of the difference spectrum reveals that the $[4Fe-4S]^{2+}$ clusters (50%; 0.50 eq of $[4Fe-4S]^{2+}$ per dimer; blue line) are converted to $[2Fe-2S]^{2+}$ clusters (25% of total iron; 0.50 eq $[2Fe-2S]^{2+}$ per dimer; green line). The missing intensity can be identified by comparison of the experimental difference spectrum with the superposition of the two quadrupole doublets (red line). The line at $-0.3$ mm/s occurs at a position typical of octahedrally nitrogen/oxygen-coordinated high spin Fe(II) (−10%; based on the intensity of this line). In addition, there is a broad, featureless absorption pointing upward. This fraction is assigned to nonspecifically bound iron because the EPR spectrum of reconstituted NStrepChlR after exposure to O$_2$ exhibits only weak features attributable to $[3Fe-4S]^+$ clusters (Fig. 9B, black line). The EPR signal with $g_{av} \approx 2.02$ amounts after integration to 0.04 spin per NStrepChlR dimer.

Taken together, the spectroscopic and analytical methods reveal that NStrepChlR reconstituted under anoxic conditions harbors 0.74 $[4Fe-4S]^{2+}$, 0.30 $[2Fe-2S]^{2+}$, and 0.01 $[3Fe-4S]^+$ clusters per dimer. Upon exposure to O$_2$ for 15 min, the majority of $[4Fe-4S]^{2+}$ (0.50 eq) are converted to $[2Fe-2S]^{2+}$ (0.50 eq), whereas the remaining two irons of the disassembled cluster are presumably released into solution because they give rise to Mössbauer features associated with high spin Fe(II) in solution and an aggregated, nonspecific form of iron.

**DISCUSSION**

In this study, we showed that the product of ORF SYNPCC7002_A1993, annotated as a putative transcriptional regulator, is an oxygen-sensitive transcriptional activator for an operon of four genes, acsF$_H$, ho2, hemN, and desF. Three of the resulting enzymes catalyze important reactions of photosynthetic pigment biosynthesis under microoxic conditions. HemN acts in heme biosynthesis and converts coproporphyrin III to protoporphyrinogen IX, AcsF$_H$ (ChlA$_H$) converts Mg-protoporphyrin IX monomethyl ester into 3,8-divinylprotochlorophyllide in chlorophyll a biosynthesis, and Ho2 oxidatively cleaves the tetrapyrrole ring of heme to produce biliverdin (7, 15, 16, 23). Under atmospheric O$_2$ levels, these same reactions are catalyzed by HemF, AcsF$_I$ (ChlA$_I$), and Ho1 (Hox), respectively. Although the exact function of desF has not yet been demonstrated, based on sequence similarity, it is likely that this protein substitutes for the essential fatty acid desaturase DesC under microoxic conditions (46). The desF gene is often absent from similar operons in other cyanobacteria (e.g. Synechocystis 6803). The paralogous enzymes encoded in such operons, which are presumably optimized to function under microoxic conditions, occur in numerous cyanobacteria. Similar gene clusters like the acsF$_H$-ho2-hemN-desF cluster of Synechococcus 7002 are present in many cyanobacterial strains from different habitats (data not shown); however, the gene composition of these clusters is variable. For example, Lepolyngbya sp. strain PCC 7376 has a similar five-gene operon that includes a psbA paralog.

A very similar MarR-type transcriptional regulator (sll1512) has recently been reported in Synechocystis 6803 (25). That study demonstrated that a constitutively activated variant of ChlR binds to the promoter sequences of acsF$_H$ (ChlA$_H$) and psbAI. Transcript levels of these genes and of hemN and ho2, the other two genes of the acsF$_H$ (ChlA$_H$) operon, are expressed at higher levels under microoxic conditions (24). In Synechococcus 7002, however, no changes of the transcript levels of photosystem-related genes and in particular of the three paralogs of psbA were observed in response to microoxic or anoxic conditions (2). Changes of the transcript level of a few other genes related to iron uptake or stress, isiA and isiB in particular, were observed in some microoxic samples (Table 3). This appears to be a secondary effect due to differences in iron availability under low O$_2$ levels because our experimental setup did not allow rigorous control of the O$_2$ level in liquid cultures. The results of our complementation experiments, however, showed
clearly that isiA and isiB are not regulated by ChlR (Table 3).

Only the four genes of the acsFII-hio2-hemN-desF operon specifically responded to changing O2 levels. MarR-type transcription regulators typically form homodimers (47), and it was proposed that ChlR of Synechocystis 6803 similarly forms a homodimer that binds to the acsFII (chlAII) promoter region when activated (25). In this study, we show experimentally that ChlR from Synechococcus 7002 forms homodimers in vitro and can activate transcription from the acsFII (chlAII) promoter of Synechocystis 6803 in E. coli in vivo and that no other cyanobacterium-specific factors are required for O2 sensing and transcription activation. By placing a yfp reporter gene under the control of the inducible acsFII (chlAII) promoter and by transferring the system into both Synechococcus 7002 and E. coli, we demonstrated that this system can be used to express genes in a manner that is solely controllable by the oxygen level.

FIGURE 11. Sequence comparison of ChlR homologs from diverse cyanobacteria. Cys residues are shaded in gray. The shaded aspartic acid residue in the Synechococcus 6803 sequence causes constitutive activation of ChlR when mutated to His (25). Hyphens denote insertions/deletions introduced to optimize the alignment. In the consensus line, stars indicate absolutely conserved residues, and colons and periods indicate conservative replacements.
This adds to a growing list of expression systems for cyanobacteria, and this system should be especially useful for controlling the expression of hydrogenase genes in cells that are fermenting or in cells that at least have minimal intracellular O₂ levels.

MarR-type regulators comprise a broad family of transcription activators and repressors that regulate a variety of stress responses and other cellular processes, and specific binding of ligands has been shown for some examples (47). The purified, reconstituted MarR-type transcription activator ChlR of *Synechococcus* 7002 harbors an Fe-S cluster as an oxygen-sensitive prosthetic group. A combination of spectroscopic and analytical methods revealed the presence of 0.74 [4Fe-4S]_2^- clusters and 0.30 [2Fe-2S]_2^- clusters, i.e. a total of 1.04 clusters per ChlR homodimer. The presence of some [2Fe-2S] clusters and a minute amount of [3Fe-4S]_2^- clusters in ChlR that had been reconstituted and purified under anoxic conditions was presumably due to minor O₂ contamination during the purification process and manipulations required to prepare the protein for spectroscopic measurements. Our findings together with the previously described homodimeric structure of other MarR-type regulators as well as the ChlR homolog of *Synechocystis* 6803 (ChlR) (25, 47) strongly suggest that one [4Fe-4S] cluster is bound per ChlR homodimer. Database searches show that ChlR-type regulators occur in numerous cyanobacteria derived from a wide variety of habitats, but ChlR homologs do not seem to occur in all cyanobacteria. MarR family members also occur in cyanobacteria that do not have *acsF-II-ho2-hemN-desF* operons, but it is presently unknown what these other transcription factors control.

If one considers all cyanobacterial MarR family members, only one Cys residue (the second cysteine; residue 14 in ChlR from *Synechococcus* 7002) is conserved (see Fig. 11). This led Aoki *et al.* (25) to suggest that this cysteine residue might form a sulfenic acid or S-thiolated derivative under oxic conditions (non-activating ChlR) that could be reduced to a free thiol under microoxic conditions (activating ChlR) (25). However, if one only considers those MarR family members that are most similar to ChlR (also restricting the analysis to organisms that have operons similar to *acsF-II-ho2-hemN-desF*), a different pattern emerges (Fig. 11). Comparison of this more limited set of ChlR homologs shows that these proteins have four conserved Cys residues; two occur near the N terminus, and two occur near the C terminus. The C-terminal pair of Cys residues always occurs in the pattern Cys-X-Cys, whereas the N-terminal pair of Cys residues occurs in the motif Cys-X₄₋₆-Cys-Pro. The N-terminal Cys motif is reminiscent of a portion of the Cys motif for [4Fe-4S] ferredoxins (i.e. Cys-X₂-Cys-X₂-Cys-X₃-Cys-Pro), and furthermore, these Cys residues occur in close prox-

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**FIGURE 12. Model for O₂-dependent regulation by ChlR.** A homodimer of ChlR lacking Fe-S clusters (A) cannot bind to the *acsF-II* promoter. After insertion of a [4Fe-4S] cluster (B) by the Fe-S cluster biogenesis machinery, ChlR can bind to its operator near the *acsF-II* promoter (C) and activate transcription (D). Exposure to oxygen (E) converts the [4Fe-4S] cluster to a [2Fe-2S] cluster, and the transcription activator loses its ability to bind to the *acsF-II* promoter (F). It is presently not clear whether an Fe-S cluster can be restored or reinserted into the inactivated protein or whether this protein turns over and must be resynthesized.
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Imarity to the N-terminal dimerization domain for MarR proteins. The C-terminal pair of Cys residues occurs in a motif that might bind a metal ion or form a disulfide bond that could respond via thioether to intracellular redox conditions. Because ChlR does not contain a Cys motif typical for Fe-S cluster ligation, we propose that the [4Fe-4S] cluster is bound at the dimer interface by two N-terminal Cys motifs. As shown here, it is very likely that the [4Fe-4S] cluster is the O$_2$-sensing component. The role of the Cys residues of Synechococcus 7002 ChlR as ligands to the [4Fe-4S] cluster is presently unresolved and must be clarified by further studies.

Upon exposure to O$_2$, the [4Fe-4S]$^{2+}$ cluster of ChlR was converted to a [2Fe-2S]$^{2+}$ cluster. The remaining two irons of the [4Fe-4S] cluster are apparently released into solution because they exhibit Mössbauer features typical of high-spin Fe(II) in solution and aggregated, nonspecific iron. This degradation mechanism is strikingly similar to the O$_2$-mediated conversion of the [4Fe-4S]$^{2+}$ cluster of the FNR transcription factor into a [2Fe-2S]$^{2+}$ cluster (48–50), which is known to be uniquely coordinated by cysteine persulfide ligands rather than cysteinate residues (51). Based on the homodimeric structure of ChlR and the observed structural changes of the Fe-S cofactor, we propose a plausible and testable model for the O$_2$-dependent transcriptional regulation of the $acsF_{II}$ promoter. The cellular Fe-S cluster biosynthesis machinery (52) inserts a [4Fe-4S] cluster into homodimeric ChlR. When the O$_2$ level is sufficiently low (microoxic conditions), the [4Fe-4S] cluster is stable (C). This conformation of the regulator allows ChlR to bind to the $acsF_{II}$ promoter and activate transcription of the $acsF_{II}$-ho2-hemN-desF operon (D). When the regulator is exposed to oxygen (E), the [4Fe-4S]$^{2+}$ cluster is degraded to a [2Fe-2S]$^{2+}$ cluster (or probably beyond), resulting in a conformational change of the homodimer (F) and causing its dissociation from the $acsF_{II}$ promoter sequence. As a consequence, transcription of the $acsF_{II}$-ho2-hemN-desF operon ceases. The fate of ChlR containing a [2Fe-2S]$^{2+}$ cluster is presently unknown. It is possible that ChlR is proteolytically degraded, or alternatively, the [4Fe-4S] cluster can be restored either by disassembly of the [2Fe-2S] cluster followed by reinsertion by the Fe-S cluster biosynthesis system for O$_2$-sensitive enzymes. This system will be particularly interesting for expression of hydrogenases or other enzymes involved in fermentative pathways.

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