Transcriptional regulators ChlR and CnfR are essential for diazotrophic growth in nonheterocystous cyanobacteria

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***Leptolyngbya boryana* (*Plectonema boryanum*) is a diazotrophic cyanobacterium lacking heterocysts. How nitrogen fixation is regulated in filamentous nonheterocystous cyanobacteria remains unclear. Here we describe a large 50-kb nitrogen fixation (*nif*) gene cluster in *L. boryana* containing 50 genes. This gene cluster contains 14 *nif* genes (*nifBSUHDKVZT* and *nifPENXW*), two genes encoding transcriptional regulators showing high similarity to ChlR (chlorophyll regulator) and PatB, three genes encoding ferredoxin, three genes encoding cytochrome oxidase subunits, and 28 genes encoding *nif*-related proteins and proteins with putative or unknown functions. Eleven mutants lacking one gene or a subset of genes were isolated. Five of them did not grow under diazotrophic conditions, including two mutants lacking the transcriptional regulators. Although the *chlR* homolog-lacking mutant showed a normal level of nitrogenase activity, various intermediates of chlorophyll biosynthesis were accumulated under microoxic conditions. The phenotype suggested that ChlR activates the expression of the genes responsible for anaerobic chlorophyll biosynthesis to support energy supply for nitrogen fixation. In another mutant lacking the *patB* homolog, no transcripts of any *nif* genes were detected under nitrogen fixation conditions, which was consistent with no activity. Constitutive expression of *patB* in a shuttle vector resulted in low but significant nitrogenase activity even under nitrate-replete conditions, suggesting that the PatB homolog is the master regulator of nitrogen fixation. We propose to rename the *patB* homolog as *cnfR*, after cyanobacterial nitrogen fixation regulator.**

anoxia-induced expression | iron–sulfur cluster

Nitrogen fixation is a process for converting atmospheric nitrogen (N_2) to ammonia, the major bioavailable nitrogen (1). Together with industrial nitrogen fixation based on the Harber–Bosch process, biological nitrogen fixation supports the bioproductivity on the earth. The enzyme responsible for nitrogen fixation is nitrogenase, which consists of two separable components: the Fe protein and MoFe protein (2). The Fe protein, a homodimer of the NifH protein, plays a role as an ATP-dependent reductase component for the MoFe protein. A [4Fe-4S] cluster is held between the homodimeric interfaces of the Fe protein. On hydrolysis of ATP, an electron of the [4Fe-4S] cluster is transferred to the MoFe protein. The electrons from the Fe protein eventually reach the catalytic site, FeMo-cofactor (FeMo-co), via an intermediate cluster, the P-cluster, and the N_2 molecule bound in the vicinity of FeMo-co is then reduced to ammonia. The metallocenters of the nitrogenase components are extremely vulnerable to oxygen. Once exposed to the air, both the components become irreversibly inactivated with half-lives in the order of several seconds to minutes (3). Thus, the process of nitrogen fixation requires an anoxic environment, and all nitrogen-fixing organisms have some mechanism for maintaining the diazotrophic cells anoxic to drive nitrogenase.

Cyanobacteria are photosynthetic prokaryotes that perform oxygenic photosynthesis similar to plants. Many cyanobacteria have the ability to fix nitrogen with nitrogenase (4). Unlike heterotrophic nitrogen-fixing bacteria, cyanobacteria have special mechanisms for protecting nitrogenase from oxygen, given that they evolve oxygen by oxygenic photosynthesis. Some filamentous cyanobacteria form heterocysts specialized for nitrogen fixation (5). The strategy of heterocyst differentiation is the spatial separation of nitrogenase from photosynthesis. On the deprivation of combined nitrogen compounds, approximately every 10th cell along the filament of vegetative cells differentiates to heterocysts within 20 h in the cyanobacterium *Anabaena* sp. PCC 7120 (*Anabaena* 7120). Heterocysts are characterized by the following properties: they have a reduced content of photosystem II, the oxygen-evolving complex (6); they develop special cell walls as a physical barrier for oxygen penetration from the environment (7); and they have high respiratory activity by cytochrome *c* oxidase to remove oxygen and produce ATP to support nitrogenase activity (8).

Many nitrogen-fixing cyanobacteria lack heterocysts. Such cyanobacteria include unicellular [*Synechococcus* (9, 10), *Gloeothece* (11), and *Cyanothece* (12)] and filamentous species [*Trichodesmium* (13) and *Leptolyngbya* (14)]. Nitrogen fixation in such species is temporally separated from oxygenic photosynthesis and regulated by circadian rhythms (9, 10, 15). In microbial mats in Yellowstone National Park, nitrogenase activity by some unicellular *Synechococcus* strains was detected only at dawn and sunset (16), supporting the circadian regulation of nitrogen fixation in field environments. In *Gloeothece*, nitrogen fixation is mainly regulated by circadian rhythm and modulated

Significance

Nitrogen fixation is a process of conversion of atmospheric nitrogen to ammonia catalyzed by nitrogenase, which is quickly inactivated by oxygen. Cyanobacteria are a group of prokaryotes that perform oxygenic photosynthesis, and many cyanobacterial species have the ability to fix nitrogen. How nitrogen fixation is coordinated with oxygenic photosynthesis remains largely unknown. Here we report two transcriptional regulators, ChlR (chlorophyll regulator) and CnfR (cyanobacterial nitrogen fixation regulator), that activate the transcription of genes responsible for anaerobic chlorophyll biosynthesis and the nitrogen fixation genes, respectively, in response to low-oxygen conditions in *Leptolyngbya boryana*, a diazotrophic cyanobacterium lacking heterocysts.

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by other factors such as fixed nitrogen amounts and environmental O_2 levels (11). *Trichodesmium* species are a group of major nitrogen fixers in the ocean. Almost all cells along with the trichome filaments fix nitrogen, mainly using a temporal separation mechanism under circadian control (17, 18). *Cyanothece* species performing nitrogen fixation under aerobic conditions have recently received attention (19). Extensive studies on transcriptomic and proteomic aspects conducted with the aim of understanding how these species combine nitrogen fixation and oxygenic photosynthesis in one cell have revealed dynamic metabolic changes resulting from complex regulatory mechanisms, including circadian control (15, 20, 21). However, the mechanisms by which genes responsible for nitrogen fixation are transcriptionally regulated under conditions that require nitrogen fixation in nonheterocystous cyanobacteria remain unknown.

Leptolyngbya boryana (formerly *Plectonema boryanum*) is a filamentous nonheterocystous nitrogen-fixing cyanobacterium that grows diazotrophically under micro-oxic conditions (14). Early studies of nitrogen fixation in *L. boryana* have indicated that the induction of nitrogenase requires both micro-oxic and nitrogen starvation conditions (14, 22, 23). Temporal separation of nitrogen fixation from photosynthesis has been observed in photosynthetic growth under micro-oxic conditions, suggesting the presence of a circadian control mechanism (24). The nucleotide sequence of *nifH* of *L. boryana* has been determined (25), and a transformation system for isolating a mutant lacking a targeted gene via electroporation has been established (26). Thus, *L. boryana* provides a promising model to investigate nitrogen fixation in cyanobacteria.

In the present study, we identified a large nitrogen fixation gene cluster in *L. boryana* strain *dg5* that contains two genes encoding transcriptional regulators showing high similarity to ChlR (chlorophyll regulator) from *Synechocystis* sp. PCC 6830 (*Synechocystis* 6803) and to PatB from *Anabaena* 7120, respectively. Our results suggest that the ChlR homolog supports nitrogen fixation by the transcriptional activation of genes responsible for chlorophyll, heme, and bilin biosynthesis under low-oxygen conditions and that the PatB homolog, cyanobacterial nitrogen fixation regulator (CnfR), plays a role as the master transcriptional regulator for the *nif* genes in this gene cluster.

Results

Nitrogen Fixation Gene Cluster. We determined the nucleotide sequence of genes encoding nitrogenase, as well as nitrogen fixation-related genes in *L. boryana* strain *dg5*. The nucleotide sequence of the 3.4-kb HindIII fragment containing *nifUHD* has been previously reported in *L. boryana* (25). Starting from this known sequence, genomic walking was performed both downstream of *nifD* and upstream of *nifU* (Fig. S1 and Table S1). The nucleotide sequence of a 50-kb region (50,712 bp) was determined. This gene region contains 50 genes and is divided into right and left parts at the intergenic region between *nifP* and *nifB* (Fig. 1 and Table S2). Right and left genes appear to be divergently transcribed from this intergenic region. The right part (Fig. 1 and Table S2, Nos. 30–50) contains the nitrogenase structural genes *nifH*, *nifD*, and *nifK*, which are flanked by four genes (*nifB*, *fdxN*, *nifS*, and *nifU*) and three genes (*nifV*, *nifZ*, and *nifT*) in upstream and downstream regions, respectively. A gene (*orf534*) encoding an XRE (xenobiotic response element) family transcriptional regulator is found in the opposite direction of *nifVZT*. The deduced amino acid sequence of this gene shows the highest similarity (58%) to that of *patB* from *Anabaena* 7120 (27). Downstream of *patB*, three genes encoding subunits of cytochrome *c* oxidase are found in the same direction as *nifHDK*. These three proteins show the highest similarity to those of the heterocyst-specific type oxidase subunits, CoxB2, CoxA2, and CoxC2, in *Anabaena* 7120 (8).

In the left part of the gene cluster (Fig. 1 and Table S2, Nos. 1–29), *nifP* (*cysE*) encoding serine *O*-acetyltransferase is located in the opposite direction and followed by nine genes: *orf84*, *dpsA*, *orf99*, *nifE*, *nifN*, *nifX*, *orf155*, *orf77*, and *nifW*. *nifEN* encodes the scaffold protein NifEN for the biosynthesis of FeMo-co. Another gene subcluster, *hesAB*, *fdxH*, *feoA*, *fdxB*, and *mop*, is found downstream of *nifW*. Three genes, *modA*, *modB*, and *modC*, encoding a molybdenum transporter are located downstream of *mop*. Three genes, *orf332*, *orf118*, and *orf136*, lie just downstream of *modABC* in the opposite direction. The amino acid sequence of *orf136* shows 55% similarity to that of *chlR* from the nondiazotrophic cyanobacterium *Synechocystis* 6803 (28). Seven genes, *pflB*, *pflA*, *adhE*, *acs*, *orf263*, *orf207*, and *xfp*, most of which encode enzymes for anaerobic metabolism, are found in the leftmost part of the left region. Compared with other nonheterocystous strains, 30 genes from *modC* to *orf206a* in the central part of the gene cluster in *L. boryana* are conserved in the *nif* gene cluster of *Cyanothece* sp. ATCC 51142 in almost the same gene order, with the exception of a single inversion of the 12-kb region from *dpsA* to *nifK* (Fig. S2). A draft genome of *L. boryana* sp. PCC 6306, a different strain of *L. boryana*, was recently reported (29) as 1 of 54 diverse cyanobacterial species to analyze cyanobacterial phylogenetic relationship. We found that the 50-kb *nif* gene cluster of *dg5* was identical to that of the draft genome of *L. boryana* sp. PCC 6306.

Isolation of 11 KO Mutants and Their Phenotypes. To determine whether the genes in this region are involved in nitrogen fixation, we isolated 11 mutants, NK1–NK11, lacking single or multiple genes (Figs. 1 and 2). We evaluated their growth under the three conditions (Fig. 2, lanes a–c) and their nitrogenase activity as acetylene reduction activity (Fig. 2, *Right*). The growth of all of

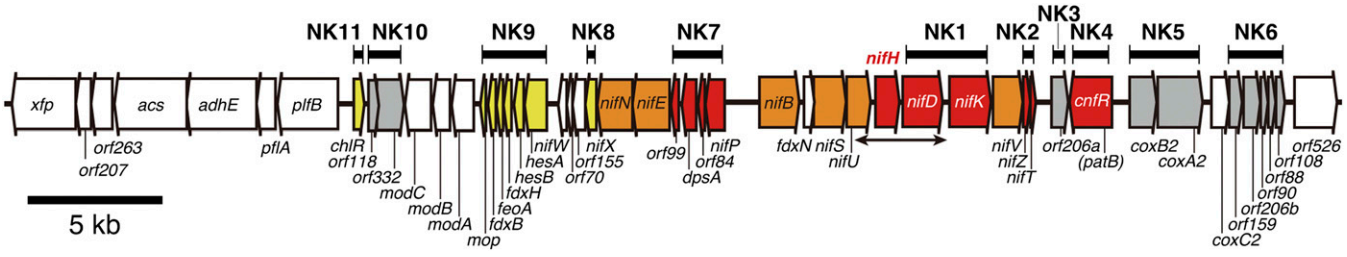

Fig. 1. Gene organization of the 50-kb nitrogen fixation gene cluster from *L. boryana*. The thick horizontal bars above the gene organization indicate the coding regions that were replaced with the kanamycin cartridge in the NK mutants. Based on the phenotype of the NK mutants, the genes are color-coded as follows: red, genes essential for nitrogen fixation; orange, genes essential for nitrogen fixation (but with no experimental evidence in *L. boryana*); yellow, genes important for nitrogen fixation; gray, genes not essential for nitrogen fixation; white, no experimental evidence available to date. It should be noted that at least one of the genes (not yet specified in this study) is essential or important for nitrogen fixation in NK2, NK7, and NK9. The thin bar with arrowheads at both ends indicates the chromosomal 3.4-kb *nifUHD* region whose nucleotide sequence has been previously reported (25).

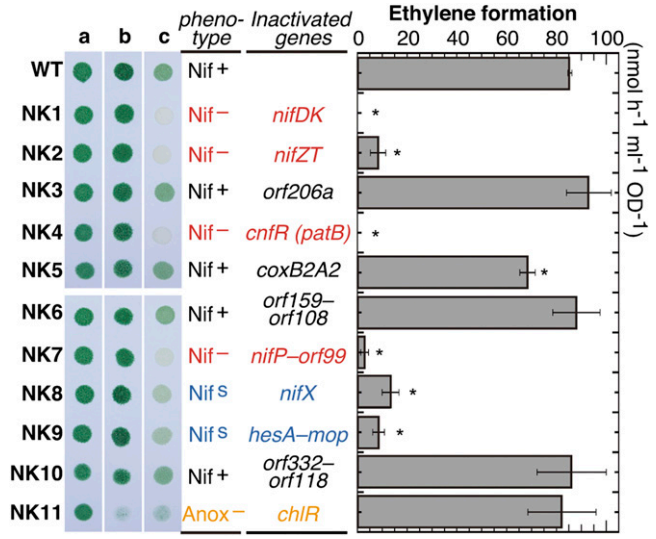

Fig. 2. Comparison of growth and acetylene reduction activity of the WT and transformants. *L. boryana* cells grown on BG-11 agar plates containing nitrate under aerobic conditions for 2 d were collected to adjust the cell density to an OD_{730} of 1.0, and aliquots (6 μL) were spotted onto new BG-11 agar plates containing nitrate (lanes a and b) or BG-11_0 without combined nitrogen (lane c), followed by growth under aerobic (lane a) or micro-oxic (lanes b and c) conditions for 4 d. Acetylene reduction activity was determined in the transformant cells. Cells grown under nitrate-replete conditions were incubated under nitrogen-fixing conditions for 12 h. Asterisks indicate values of ethylene formation rates that are statistically different from those of the WT ($n = 3$).

the mutants was the same as that of the WT in nitrate-replete agar medium under aerobic conditions (Fig. 2, lane a). On the nitrate-replete agar medium under micro-oxic conditions, all of the mutants showed normal growth except for a *chlR* homolog-lacking mutant, NK11, which showed poor growth with abnormal bluish color. Under conditions for nitrogen fixation (nitrate-depleted/micro-oxic), the four mutants NK3, NK5, NK6, and NK10 grew normally like the WT (Nif^+). These results suggested that all of the genes disrupted in these mutants are not important for nitrogen fixation.

The growth of NK8 (Δ*nifX*) and NK9 (Δ*hesAB-fdxH-feoA-fdxB-mop*) was markedly slower than that of the WT under nitrogen-fixing conditions (Nif^S). This slow growth was accompanied by a marked decrease in acetylene reduction activity in NK8 (16%) and NK9 (10%). This phenotype suggested that NifX plays an important, although not essential, role in nitrogen fixation and that one or some genes disrupted in NK9 also take part in nitrogen fixation. All six genes, *hesA*, *hesB*, *fdxH*, *feoA*, *fdxB*, and *mop*, are probably involved in nitrogen fixation. Further experiments are in progress to ascertain the contribution of the individual genes for nitrogen fixation.

NK1 (Δ*nifDK*), NK2 (Δ*nifZT*), NK4 (Δ*patB*), and NK7 (Δ*nifP-orf84-dpsA-orf99*) were completely deficient in diazotrophic growth ability (Nif^-), and NK11 (Δ*chlR*) showed a growth defect under anoxic conditions ($Anox^-$). The growth defect of NK1 in diazotrophic conditions was as expected. NK2 showed only ~10% of acetylene reduction activity, suggesting that either or both of the small proteins, NifZ and NifT, are essential for the maximal activity of nitrogenase. Interestingly, similar to NK1, NK4 showed no acetylene reduction activity. The transcriptional regulator PatB homolog is probably essential for the induction of *nif* genes in response to the depletion of combined nitrogen (see below). NK7 lacks *nifP*, *dpsA*, and two unknown genes: *orf84* and *orf99*. The deletion of the four genes caused a severe decrease (3%) in acetylene reduction activity. Despite growth defect under nitrogen fixation conditions, NK11 showed a normal level of acetylene reduction activity. The common growth defect under micro-oxic conditions and the normal level of acetylene reduction activity strongly suggested that the ChlR homolog (Orf136) is not directly involved in nitrogen fixation.

Characterization of NK11 (Δ*chlR*). *chlR* encodes a MarR-type transcriptional activator for the low-oxygen induction of a small operon, $chlA_{II}$-*ho2-hemN,* in *Synechocystis* 6803 (28). The Δ*chlR* mutant of *Synechocystis* 6803 shows poor growth under micro-oxic conditions with a very low level of Chl because the $chlA_{II}$ operon is not expressed and Chl biosynthesis is delayed in the steps of oxygen-dependent reactions. The three genes $chlA_{II}$, *ho2*, and *hemN* encode Mg-protoporphyrin IX monomethylester (MPE) cyclase, heme oxygenase 2, and coproporphyrinogen III oxidase, respectively, and all of the enzymes complement the activities of the three oxygen-dependent reactions that are normally catalyzed by $ChlA_I$ (30), HO1 (31), and HemF (32), respectively, under micro-oxic conditions. To confirm the function of the *chlR*-homolog, we determined the Chl content and analyzed the pigment composition by HPLC. The Chl content of NK11 cells incubated under micro-oxic conditions for 7 d was only ~20% that of the WT, in contrast to the normal content under aerobic conditions (Fig. 3*A*, *Inset*). This marked decrease in the Chl content is consistent with the abnormal blue color derived from phycobiliproteins (28) exhibited by colonies of NK11. As shown in the HPLC profile (Fig. 3*A*), NK11 cells accumulated various intermediates of Chl biosynthesis. This phenotype was substantially the same as that of Δ*chlR* of *Synechocystis* 6803 (28, 33).

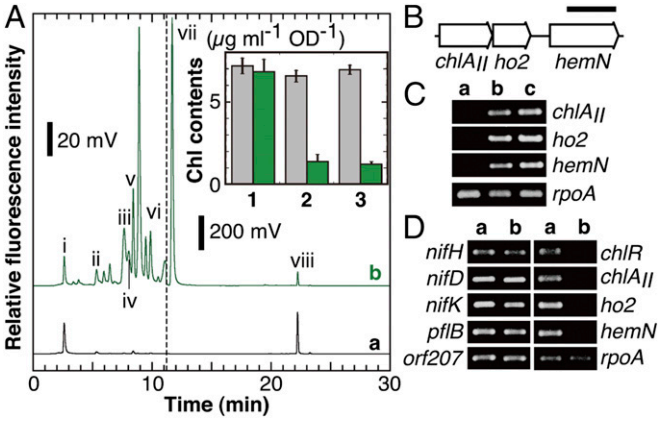

Fig. 3. Phenotype analysis of NK11. (*A*) WT and NK11 cells were incubated under micro-oxic conditions for 7 d. Aliquots (100 μL with an OD_{730} of 100) of the cell suspension were mixed with 900 μL of ice-cold methanol for HPLC analysis. The elution of pigments was monitored by fluorescence emission at 630 nm with excitation at 400 nm (*a*, WT; *b*, NK11). The elution profiles from 0 to 11.2 min are enlarged 10 times to emphasize small peaks (see the scale bars). Peaks *i* to *viii* were previously assigned as coproporphyrin III (*i*), Mg-protoporphyrin IX (*ii*), divinyl protochlorophyllide (*iii*), monovinyl protochlorophyllide (*iv*), MPE (*v*), protoporphyrin IX (*vi*), demetallated MPE (*vii*), and Chl *a* (*viii*), respectively. (*Inset*) Comparison of Chl levels of the WT and NK11. WT (gray bars) and NK11 (green bars) cells were grown in BG-11 agar plates containing nitrate (lanes 1 and 2) or BG-11_0 without combined nitrogen (lane 3) under aerobic (lane 1) or micro-oxic (lanes 2 and 3) conditions ($n = 3$). (*B*) The gene organization of the three genes, $chlA_{II}$, *ho2*, and *hemN*, forming a small gene cluster. (Scale bar, 1 kb.) (*C*) Transcript profiles by RT-PCR for the three genes. WT cells were grown under nitrate-replete/aerobic (lane a), nitrate-replete/micro-oxic (lane b), and nitrate-depleted/micro-oxic (lane c) conditions, and RNA was extracted from the cells. *rpoA* was used as an internal control. (*D*) Transcript profiles by RT-PCR for *nifHDK* and the six genes in WT and NK11 cells. WT (lane a) and NK11 (lane b) cells were grown under nitrogen-fixing conditions, and RNA was extracted from the cells.

The *chlA*$_{II}$*-ho2-hemN* operon is conserved in the draft genome sequence of *L. boryana* stain *dg5* (Fig. 3*B*). As observed in *Synechocystis* 6803, the three genes were only expressed in cells grown under micro-oxic conditions (Fig. 3*C*). Under nitrate-depleted micro-oxic conditions, no transcripts were detected for the *chlA*$_{II}$*-ho2-hemN* operon in NK11, whereas the transcripts for *nifHDK* were normally detected as in the WT (Fig. 3*D*). We also examined whether the *pflB* subcluster (*pflBA*, *adhE*, *acs*, *orf263*, *orf207*, and *xfp*) located next to *chlR* is regulated by ChlR. The seven genes, including *pflB* and *orf207* as representatives of the *pflB* subcluster (Fig. 3*D*), were expressed in NK11 as observed in the WT, indicating that ChlR does not regulate their expression. These results indicated that *orf136* is the *chlR* ortholog in *L. boryana*.

Characterization of NK4 (*ΔpatB*). NK4 showed the most prominent Nif$^-$ phenotype comparable to NK1 (Fig. 2). To examine whether the PatB-homologous protein regulates the expression of *nif* genes, we compared the transcript profiles between the WT and NK4 (Fig. 4*A* and Fig. S3). No transcripts for any *nif* genes were detected in NK4 cells incubated under nitrogen-fixing conditions, an observation that explains why NK4 showed no detectable nitrogenase activity (Fig. 2). The expression of nine genes from *chlR* to *xfp* in the leftmost part and *orf526* was not affected by the loss of the *patB* homolog. This result suggests that the PatB homolog activates the transcription of the *nif* genes in this cluster. RT-PCR in the WT indicated that the *patB* homolog was predominantly expressed under nitrate-depleted conditions, irrespective of aerobic or micro-oxic conditions (Fig. 4*A*).

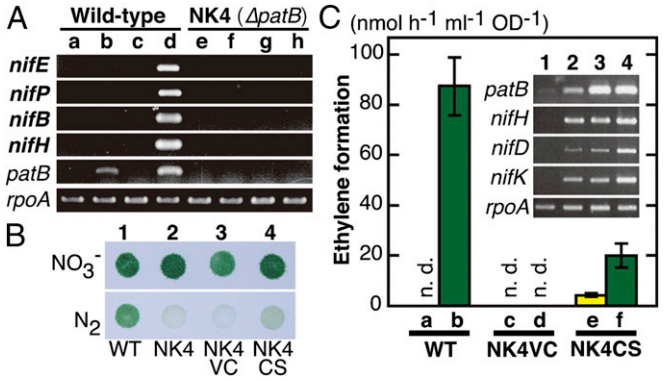

Fig. 4. Transcript levels in the WT and NK4 and complementation analyses. (*A*) Transcript profiles by RT-PCR. WT (lanes a–d) and NK4 (lanes e–h) cells grown on BG-11 agar plates under aerobic conditions were transferred to new BG-11 agar plates containing nitrate (lanes a, c, e, and g) or BG-11$_0$ (lanes b, d, f, and h), followed by incubation under aerobic (lanes a, b, e, and f) or micro-oxic (lanes c, d, g, and h) conditions for 6 h. RNA was extracted from the cells to prepare cDNA, and the transcript levels were estimated by RT-PCR. (*B*) NK4VC and NK4CS are NK4 derivatives harboring the shuttle vectors pPBH202 (vector control) (38) and pPBHPatBS containing *strep-patB* under the control of the T5 promoter, respectively. Cells of the WT (lane 1), NK4 (lane 2), NK4VC (lane 3), and NK4CS (lane 4) grown on nitrate-replete BG-11 agar plates under aerobic conditions were spotted on new nitrate-replete BG-11 agar plates (NO_3^-) or BG-11$_0$ (N_2), followed by incubation under micro-oxic conditions for 4 d. (*C*) Acetylene reduction activity was determined in the transformant cells. Cells grown under nitrate-replete conditions were incubated under nitrate-replete (yellow bars; lanes a, c, and e) and nitrogen-fixing (green bars; lanes b, d, and f) conditions for 12 h. n.d., not detected. (*Inset*) The transcript levels of *patB* and *nifHDK* were estimated by RT-PCR. WT (lanes 1 and 2) and NK4CS (lanes 3–4) cells grown on BG-11 agar plates under aerobic conditions were transferred to new BG-11 agar plates containing nitrate (lanes 1 and 3) or BG-11$_0$ (lanes 2 and 4) under micro-oxic conditions for 12 h. The transcript levels were estimated by RT-PCR as shown in *A*.

To examine whether constitutive expression of the *patB* homolog induces the *nif* genes even under nitrogenase-repressed conditions, we constructed a shuttle vector carrying a fusion gene encoding Strep-tagged PatB-homolog under the control of an *Escherichia coli* T5 promoter, and transformed NK4 to isolate NK4CS (Fig. 4 *B* and *C*). NK4CS restored diazotrophic growth under nitrogen-fixing conditions, although the growth was slower than that of the WT (Fig. 4*B*). In addition, ∼20% activity of that in the WT was restored in NK4CS (Fig. 4*C*, lane f). Furthermore, low but significant nitrogenase activity was detected in NK4CS, although the cells grew in the presence of nitrate (Fig. 4*C*, lane e). The transcript levels of the *patB* homolog and *nifHDK* are shown in the *Inset* to Fig. 4*C*. The transcript levels of the *patB* homolog (lanes 3 and 4) were much higher than that of WT under nitrate-depleted conditions (lane 2), and their levels were similar in both nitrate-replete and nitrate-depleted conditions, indicating that the T5-promoter regulation brought about overexpression of the *patB* homolog in NK4CS. The *nifHDK* transcripts, as representatives of the *nif* genes, were higher than those of WT under nitrate-depleted conditions (compare lanes 4 and 2). Furthermore, the *nifHDK* transcripts were detected in the nitrate-replete conditions in almost the same levels as those of WT under nitrate-depleted conditions (compare lanes 3 and 2), which was consistent with nitrogenase activity in nitrate-replete conditions (lane e), albeit at a relatively low level. These results strongly suggested that the PatB homolog is the master transcriptional regulator for nitrogen fixation in *L. boryana*.

Discussion

Cyanobacteria have developed various regulatory mechanisms to drive nitrogenase together with oxygenic photosynthesis during evolution. Heterocyst formation is the most elaborate mechanism for separating the nitrogen fixation process spatially from vegetative photosynthetic cells (5). However, molecular genetic analysis of nitrogen fixation in heterocystous strains is often complicated by a large number of genes for heterocyst differentiation and the synthesis of polysaccharide and glycolipid layers, which are not directly associated with the nitrogen fixation process itself. Therefore, nonheterocystous strains provide better systems for focusing only on nitrogen fixation. Among nonheterocystous cyanobacteria, some *Cyanothece* strains have been studied by a combination of transcriptomic and proteomic analyses (20, 21). However, the lack of an efficient transformation system has hindered the identification of the detailed mechanism underlying the regulation of nitrogen fixation (34). In the present study, we isolated 11 mutants with an efficient transformation system using the nonheterocystous strain *L. boryana*. A significant effect on diazotrophic growth was observed in seven of these mutants. This result demonstrated that *L. boryana* is a very useful model cyanobacterium for investigating the regulatory mechanism of nitrogen fixation in molecular biological aspects. Because multiple genes were disrupted in most mutants, studies are ongoing to identify the genes that are responsible for the Nif$^-$ or Nif^S phenotype using a complementation system with a shuttle vector.

NK11 showed a unique phenotype: poor growth under micro-oxic conditions (Anox$^-$) and normal activity of nitrogenase, distinct from other Nif$^-$ mutants (Fig. 2). The growth defect confirmed the critical role of a continuous supply of tetrapyrrole pigments for photosynthesis and respiration to support nitrogenase activity under micro-oxic conditions (Fig. 5). The phenotype of NK11 strongly suggested that *orf136* is the *L. boryana* ortholog of the *Synechocystis* 6803 *chlR* gene (28). The diazotrophic growth defect despite of normal nitrogenase activity in NK11 can be explained as follows. Cells used for the acetylene reduction assay were precultured in nitrate-replete media under aerobic conditions, and the cells were exposed to nitrogen-fixing conditions (nitrate-depleted/micro-oxic) for just 12 h before the

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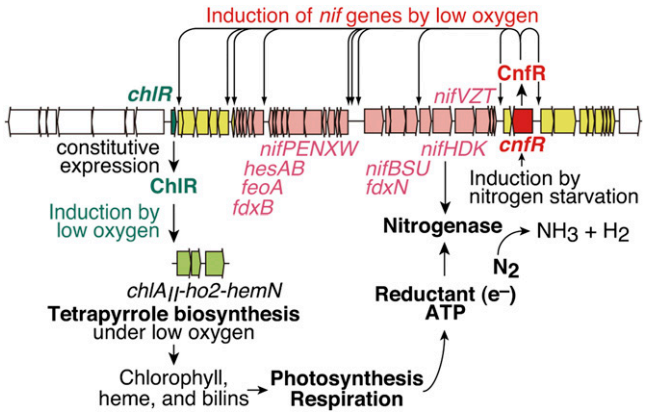

Fig. 5. A working model of ChlR and CnfR in the regulatory circuits of the *nif* gene cluster in *L. boryana*. *chlR* (shown in green) is expressed in a constitutive manner, and the ChlR protein is converted to an active form on exposure to low-oxygen environments, and it activates the expression of the small gene cluster *chlA$_{II}$-ho2-hemN* (shown in light green) in the other chromosomal locus. Thus, the three newly synthesized enzymes, ChlA$_{II}$, HO2, and HemN, bypass the oxygen-dependent reactions in tetrapyrrole biosynthesis to maintain a constant supply of Chl, heme, and bilin pigments even under micro-oxic conditions. These pigments support activities of photosynthesis and respiration to supply reductants and ATP in an efficient manner to drive nitrogenase. *cnfR* (shown in red) is induced in response to nitrogen starvation, and the CnfR protein is converted to an active form when the oxygen level becomes low enough to drive nitrogenase. Following this, the active form of CnfR activates the transcription of the *nif* genes (shown in pink) to produce active nitrogenase. Some genes shown in yellow are also regulated by some other regulatory mechanisms while they are still under the control of CnfR.

assay. The contents of preexisting Chl, bilins, and heme in the cells were probably high enough to produce and drive nitrogenase in such a short time. However, the contents of Chl, bilins, and heme were decreased due to dilution by cell divisions and eventually became too low to support nitrogenase and various cellular activities resulting in growth defect.

The PatB-homologous protein (534 amino acid residues) has the N-terminal 2 Cys motifs (CxxCxxCxxxCP and CxxCx$_8$CxxxC) that are found in a bacterial-type ferredoxin to hold two [4Fe-4S] clusters and the DNA-binding motif in the C-terminal portion. This architecture is conserved in all PatB homologs in cyanobacteria. In *Anabaena* 7120, *patB* is specifically expressed in heterocysts, and a *patB*-lacking mutant showed poor growth under nitrogen-fixing conditions that was accompanied by abnormal heterocyst differentiation pattern in the form of frequent multiple contiguous heterocysts (35). In *L. boryana*, the *patB* homolog is expressed in response to the deprivation of combined nitrogen (Fig. 4*A*). No transcripts for the *nif* genes were detected in NK4 (Fig. 4*A* and Fig. S3), a finding consistent with the total loss of acetylene reduction activity (Fig. 2). Even under nitrate-replete conditions, significant nitrogenase activity was detected in NK4CS (Fig. 4*C*, lane e), which is an NK4-derivative carrying an extra *strep-patB* gene in the plasmid. These results strongly suggest that the *patB* homolog encodes the master transcriptional activator for nitrogen fixation in *L. boryana*. We propose to rename the *patB* homolog as *cnfR*, after cyanobacterial nitrogen fixation regulator.

The constitutive overexpression of *cnfR* in the shuttle vector resulted in partial complementation of diazotrophic growth and nitrogenase activity (Fig. 4 *B* and *C*). A possibility that an N-terminal fusion with Strep-tag (Strep-CnfR) may lower the activity of CnfR can be excluded because the transcript levels of *nifHDK* were almost the same as those of WT or even higher in NK4CS (Fig. 4*C*, *Inset*). Two possibilities may be considered for the partial complementation. One is a polar effect caused by the kanamycin-resistance gene (Km^R) cartridge. Because the Km^R cartridge does not carry any terminator sequences in both ends, the transcription originating from the cartridge may produce longer transcripts functioning as antisense RNA to interfere with the expression of the contiguous genes *ORF206a* and *nifVZT*. Second, the constitutive overexpression of *cnfR* driven by the T5 promoter may cause some side effects on the cells. Irrespective of the reasons for the partial complementation, the appearance of nitrogenase activity under nitrate-replete conditions indicates that all of the genes required for the production of active nitrogenase are expressed (exemplified by *nifHDK*; Fig. 3*C*, *Inset*) even under conditions in which these *nif* genes are repressed in WT cells, a finding that strongly supports the assignment of CnfR as the master regulator.

The nitrogenase activity of NK4CS under the nitrate-replete conditions was much lower (~20%) than that of cells under nitrate-depleted conditions (Fig. 4*C*, lanes e and f), which was consistent with the difference between transcript levels of *nifHDK* under nitrate-replete and depleted conditions (Fig. 4*C*, *Inset*, lanes 3 and 4). Despite the same levels of the *cnfR* transcript, the lower transcriptional levels of *nifHDK* under nitrate-replete conditions imply two possibilities. One is that the activity of CnfR is regulated at the posttranscriptional level in response to the presence of nitrate or some other combined nitrogen form. The other is that other factors are also involved in the negative regulation of *nif* genes in response to the cellular nitrogen status.

Based on the results and features of the CnfR amino acid sequence, we hypothesize the following regulatory mechanism of the *nif* genes by CnfR (Fig. 5): the transcription of *cnfR* is induced in response to nitrogen starvation (Fig. 4*A*). CnfR is maintained as an inactive form under aerobic conditions. Once the cellular oxygen level becomes low enough to drive nitrogenase, CnfR is converted to an active form to activate the transcription of the target *nif* genes to produce active nitrogenase. Two [4Fe-4S] clusters in the N-terminal domain may contribute in sensing the cellular oxygen level. This hypothesis is in accordance with the requirement of two conditions, nitrogen starvation and micro-oxic conditions, for the expression of nitrogen fixation. Further studies of the mechanism by which *cnfR* is induced in response to nitrogen starvation and of biochemical features of CnfR are required to support this hypothesis.

The gene arrangement of subcluster *nifB-fdxN-nifSUHDK* is conserved in heterocystous cyanobacteria such as *Anabaena* 7120 and *Anabaena variabilis* ATCC 29413. Ungerer et al. (36) reported that the high expression of *nifH1* is supported by two promoters: a strong one is located upstream of *nifB1*, and the other weak one is located in the coding region of *nifU1* in *A. variabilis*. In addition, they pointed out that a secondary structure of 5′ UTR just upstream of *nifH1* might contribute to stabilize the processed *nifH1* transcript. We previously identified a monocistronic *nifH* transcript in diazotrophically grown *L. boryana* cells by Northern blot analysis (25). Short inverted repeats forming similar secondary structures in the 5′ and 3′ UTRs of *nifH* of *L. boryana* (25) might stabilize the *nifH* transcript contributing higher levels of the monocistronic *nifH* transcript than those of other *nif* genes similar to *A. variabilis*. In *Anabaena* 7120, Suzuki et al. (37) reported that AnCrpA (*Anabaena* cAMP receptor protein) is involved in a positive regulation of the *nif* and *cox* genes, which was supported by microarray data and specific binding of AnCrpA to the 5′ upstream regions of *nifB*, *hesA*, and *coxB* in the presence of cAMP. In the draft genome of *L. boryana* strain *dg5*, there is a probable ortholog of AnCrpA (76% identity). Thus, the *nif* gene cluster of *L. boryana* may be also regulated by some other mechanisms common to heterocystous strains such as CrpA in addition to the CnfR system.

Nitrogen fixation in *L. boryana* is directly regulated by the action of CnfR in transcription of *nif* genes and indirectly by the action of ChlR in the Chl supply under micro-oxic conditions. ChlR contributes not only to maintain the photosynthesis activity but also to enhance respiratory activity by the inducible cytochrome oxidase encoded by *coxB2A2C2* for protecting nitrogenase from oxygen damage. Nitrogen fixation is separated from oxygenic photosynthesis in a temporal manner in nonheterocystous cyanobacteria (9, 10). The two regulatory proteins ChlR and CnfR would play critical roles in maintaining circadian outputs in response to the cellular oxygen levels to separate these incompatible processes. How ChlR and CnfR sense the cellular oxygen level is of particular interest for future studies of cyanobacterial nitrogen fixation.

Materials and Methods

The *L. boryana* (formerly *P. boryanum*) strain *dg5* and mutants were cultivated on BG-11 medium. For nitrogen deprivation treatment, BG-11_0 medium with no combined nitrogen sources was used. For growth under micro-oxic conditions, agar plates were incubated in an anaerobic jar (BBL GasPak anaerobic systems; BD Biosciences) with a sachet for producing anoxic conditions (Gas Generating Kit Anaerobic System; Oxoid) (28) under light intensity of 40 $\mu mol_{photon} \cdot m^{-2} \cdot s^{-1}$ for 6 h to 4 d. Preparation of genomic DNA, gene walking, construction of plasmids, preparation of RNA, RT-PCR, and pigment analysis are described in *SI Materials and Methods* (Tables S3 and S4). For the induction of nitrogenase, the cells were transferred onto BG-11_0 agar plates and were incubated in an anaerobic jar in the light for 6–12 h. Nitrogenase activity was assayed by acetylene reduction, as described in *SI Materials and Methods*. The nucleotide sequences of the 50-kb *nif* gene cluster, the *chlA*$_{II}$-*ho2-hemN* gene cluster, and *rpoA* have been deposited in GenBank under the accession numbers of AB808482, AB808629, and AB808630, respectively.

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