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A Novel Nitrite Reductase Gene from the Cyanobacterium *Plectonema boryanum*

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The gene (*nirA*) for nitrite reductase was cloned from the nonheterocystous, filamentous cyanobacterium *Plectonema boryanum*. The predicted protein consists of 654 amino acids and has a calculated molecular weight of 72,135. The deduced amino acid sequence from positions 1 to 511 is strongly similar to the entire sequence of the ferredoxin-dependent nitrite reductases from other phototrophs, while the remainder of the protein is unique to the *Plectonema* nitrite reductase. The C-terminal portion of the protein (amino acids 584 to 654) is 30 to 35% identical to [2Fe-2S] ferredoxins from higher plants and cyanobacteria, with all of the four Cys residues involved in binding of the [2Fe-2S] cluster in the ferredoxins being conserved. Immunoblotting analysis of the extracts of *P. boryanum* cells showed that the NirA polypeptide has an apparent molecular mass of 75 kDa. An insertional mutant of *nirA* lacked the 75-kDa polypeptide, had no nitrite reductase activity, and failed to grow on nitrate and nitrite, indicating that the novel *nirA* is the sole nitrite reductase gene in *P. boryanum* and that the NirA polypeptide with the ferredoxin-like domain is the apoprotein of the functional nitrite reductase. As in *Synechococcus* sp. strain PCC7942, *nirA* is the first gene of a large transcription unit (>7 kb in size) and is repressed by ammonium and derepressed simply by deprivation of ammonium from the medium. The development of nitrite reductase activity was, however, found to require the presence of nitrate in the medium.

Nitrate is the major source of nitrogen for photosynthetic organisms such as plants, algae, and cyanobacteria (15, 16). It is transported into the cells by an active transport system and reduced to nitrite by nitrate reductase (NR). Nitrite is further reduced to ammonium by nitrite reductase (NiR), and the resulting ammonium is fixed as the amide group of Gln by glutamine synthetase. In plant cells, NR and NiR are located in the cytoplasm and the chloroplast, respectively (7), while in cyanobacteria, NR and NiR are located in the same compartment, the cytoplasm of the prokaryotic cell (15). In accordance with the view that the chloroplast originated from a prokaryotic endosymbiont similar to cyanobacteria (10, 45), the cyanobacterial and plant NiRs are both ferredoxin-dependent enzymes and are homologous to each other (27, 42). By contrast, the ferredoxin-dependent NR of cyanobacteria (3, 22) is distinct from the pyridine nucleotide-dependent NRs of plants and eukaryotic algae (7).

In the unicellular non-nitrogen-fixing cyanobacterium *Synechococcus* sp. strain PCC7942, the gene encoding NiR (*nirA*) (27, 42) is cotranscribed with the genes encoding the nitrate transporter (*nrtABCD*) (33–35) and NR (*narB*) (3, 22) as an *nirA-nrtABCD-narB* operon (42). Transcription from the operon is activated upon removal of ammonium from the medium or upon inhibition of ammonium fixation, showing no requirements for the presence of nitrate (42). In filamentous nitrogen-fixing cyanobacteria such as *Nostoc* sp. strain PCC6719, *Anabaena* sp. strain PCC7119, and *Anabaena variabilis* ATCC 29413, which develop heterocysts for dinitrogen fixation in the absence of combined nitrogen, expression of NR activity requires not only the absence of ammonium but also the presence of nitrate (17, 18). Nitrate is hence thought to act as an inducer of the expression of the nitrate assimilation activities in these cyanobacteria (18), although it is not known whether nitrate is required for the transcription of the genes involved.

Plectonema boryanum is a filamentous cyanobacterium belonging to section III (38), members of which perform dinitrogen fixation without forming heterocysts. Unlike the heterocyst-forming strains that perform dinitrogen fixation under aerobic conditions, *P. boryanum* requires low oxygen tension, in addition to the absence of combined nitrogen, for expression of the dinitrogen fixation system (32, 37). Regulation of the nitrate assimilation system in response to changes in oxygen tension and nitrate availability, on the other hand, has not yet been studied in detail. As an initial step in the investigation of the regulation of the nitrate assimilation system in this cyanobacterium at the level of gene expression, we cloned the gene for ferredoxin-dependent NiR and found that the deduced NiR has a large C-terminal extension that has homology to the plant-type ferredoxin. The novel protein is shown to be the sole functional NiR in the cyanobacterium by insertional inactivation of the gene and immunoblotting analysis of the protein.

MATERIALS AND METHODS

Strains and growth conditions. Cells of *P. boryanum* IAM-M101 (from the Algal Collection of the Institute of Applied Microbiology, University of Tokyo) were grown photoautotrophically at 30°C under continuous illumination provided by fluorescent lamps (70 microeinsteins m^{-2} s^{-1}). The basal medium used was a nitrogen-free medium obtained by replacing $NaNO_3$, $Co(NO_3)_2$, and ferric ammonium citrate in BG11 medium (41) with NaCl, $CoCl_2$, and ferric citrate, respectively. Ammonium-containing medium and nitrate-containing medium were prepared by adding 3.75 mM $(NH_4)_2SO_4$ and 15 mM KNO_3, respectively, to the basal medium, unless otherwise stated. All media were buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 8.0). When appropriate, kanamycin was added to the media at 10 μg/ml. The cultures were aerated with 2% (vol/vol) CO_2 in air. *Escherichia coli* DH5α

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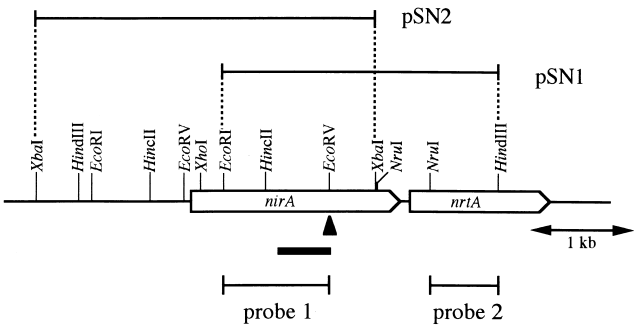

FIG. 1. Restriction map of the *nirA-nrtA* region of genomic DNA from *P. boryanum*. Above the map, the fragments of genomic DNA cloned into pSN1 and pSN2 are shown. The *Eco*RV site where an insertion was made in the mutant PN1 is indicated by an arrowhead. The closed bar below the map indicates the PCR product obtained using the degenerate oligonucleotides (see text). The probes used for Northern hybridization analysis are also shown (probes 1 and 2).

(Bethesda Research Laboratories) and JM105, used as hosts for plasmid constructions and M13 vectors, respectively, were grown on LB medium supplemented with ampicillin (50 μg/ml) and/or kanamycin (50 μg/ml) when appropriate. *E. coli* M15[pREP4] (Qiagen), used for expression of cloned *nirA*, was grown in 2× YT medium supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml).

For induction of the transcription of *nirA*, cells of *P. boryanum* grown in a medium containing 3.75 mM $(NH_4)_2SO_4$ to the mid-logarithmic phase of growth were collected by centrifugation at 5,000 × *g* for 5 min at 25°C and washed twice with the nitrogen-free medium by resuspension and recentrifugation. The washed cells were inoculated either into the nitrogen-free medium or into a medium containing 15 mM KNO_3 and then incubated at 30°C under continuous illumination as described above.

Cloning and DNA sequence determination of *nirA*. A 0.5-kbp DNA fragment from the chromosomal DNA of *P. boryanum* was amplified by PCR, using degenerate oligonucleotides synthesized according to the amino acid sequences conserved in NiRs from higher plants and the cyanobacterium *Synechococcus* sp. strain PCC7942 (see below) and shown to encode an amino acid sequence similar to those of known NiRs, as confirmed by cloning and sequencing. This PCR fragment was used to isolate clones from a library of genomic DNA of *P. boryanum* that was constructed by ligating an *Eco*RI-*Hin*dIII digest of the genomic DNA in pUC19. The plasmid purified from selected clones (pSN1; Fig. 1) was found to contain a 2.6-kbp fragment of *P. boryanum* DNA carrying an open reading frame of 1,671 bases that was strongly similar to *nirA* of *Synechococcus* sp. strain PCC7942. Since the putative *nirA* gene lacked its 5′ portion, the remainder of the gene was cloned from another library of genomic DNA constructed by ligating an *Xba*I digest of the genomic DNA in pUC19, using a 1-kbp *Eco*RI-*Eco*RV fragment of the insert in pSN1 as the probe (pSN2; Fig. 1).

For nucleotide sequence analysis, various restriction fragments of the inserts in pSN1 and pSN2 were subcloned into M13 mp18/19, and nested deletions were generated by using an exonuclease III-mung bean nuclease system (39). Single-stranded DNA templates were prepared as described by Vieira and Messing (43) and sequenced by the dideoxy-chain termination method (40). Both DNA strands were sequenced completely with no ambiguities. Database homology searches were performed with the BLAST program (2).

Insertional inactivation of *nirA*. For insertional inactivation of *nirA*, a 1.4-kbp *Eco*RI-*Xba*I fragment of the gene was cloned into pUC19. A kanamycin resistance gene cartridge, C.K1 (11), was inserted into the *Eco*RV site in the cloned *nirA* fragment. A 2.5-kbp *Pst*I fragment of pRL250 (6), carrying the conditionally lethal gene *sacB*, was subsequently ligated into the *Nde*I site in the vector portion of the plasmid. The resulting plasmid was introduced into the cells of *P. boryanum* by electroporation (14). From the kanamycin-resistant transformants obtained, the double recombinants carrying the interrupted *nirA* gene were selected on solid medium containing 5% (wt/vol) sucrose, which inhibits the growth of the single recombinants having *sacB* integrated in the genomic DNA (6). Genomic DNA was isolated from the selected clones and analyzed by PCR to confirm the presence and position of the kanamycin resistance gene.

DNA isolation and PCR. Chromosomal DNA was extracted and purified from the wild-type and mutant cells of *P. boryanum* as described by Williams (46). PCR was carried out on approximately 30 ng of chromosomal DNA. For amplification of a *nirA* fragment of *P. boryanum* for the purpose of gene cloning, degenerate oligonucleotides were synthesized according to the amino acid sequences conserved in NiRs from the cyanobacterium *Synechococcus* sp. strain PCC7942 (27, 42), spinach (5), maize (24) birch (13), and tobacco (Nir1 and Nir3) (21): 5′-ATGTGGTT(AG)AT(CT)GA(CT)GA-3′ and 5′-ATGTGGCT (AGCT)AT(CT)GA(CT)GA-3′ for MWLIDE (sense primers), and 5′-GG(AG) CA(AGCT)CC(AGCT)GTCCA(AG)TG-3′ for HWTGCP (antisense primer). For analysis of genomic DNA from the *nirA* insertional mutant, a pair of *nirA*-specific primers was synthesized on the basis of the sequence shown in Fig. 2: sequence from bases 1624 to 1642 and complementary sequence from bases 1871 to 1889.

RNA isolation and analysis. Total RNA was extracted and purified from cells of *P. boryanum* by the method of Aiba et al. (1). For Northern (RNA) hybridization analysis, RNA samples (10 μg of each) were denatured by treatment with formamide, fractionated by electrophoresis on 1.2% agarose gels that contained formaldehyde, and transferred to positively charged nylon membranes (Hybond N+; Amersham). For dot hybridization analysis, 0.5-, 1-, and 2-μg aliquots of each of the denatured RNA samples were spotted on the nylon membranes, using a dot blot apparatus. The blots were allowed to hybridize with the following probes as described by Church and Gilbert (8); a 1-kbp *Eco*RI-*Eco*RV fragment of *nirA* (probe 1) and a 0.7-kbp *Nru*I-*Hin*dIII fragment of the putative *nrtA* gene (probe 2). The double-stranded DNA probes were labeled with ^{32}P as described by Feinberg and Vogelstein (12). The hybridization signals were detected by autoradiography on X-ray film. The radioactivity of the RNA dots was quantified with a Bio-Image analyzer (Fuji Photo Film).

Preparation of antibody against the NiR polypeptide and immunoblotting analysis. The 0.25-kbp *Xho*I-*Eco*RI fragment of pSN2 and the 2.6-kbp *Eco*RI-*Hin*dIII fragment of pSN1 (Fig. 1) were cloned tandemly between the *Sal*I and *Hin*dIII sites in the polylinker of the expression vector pQE-31 (Qiagen). The resulting plasmid carried a chimeric gene encoding a truncated NirA (amino acids 30 to 654) fused to an amino acid segment carrying six consecutive His residues. The plasmid was transformed into *E. coli* M15 (44) containing the *laq*I^q repressor plasmid pREP4 (Qiagen). Expression from the vector was induced by 1 mM isopropylthiogalactopyranoside (IPTG). The protein with the His_6 affinity tag was purified on Ni^{2+}-nitrilotriacetic acid resin (20) and used for raising antibodies in mice. For immunoblotting analysis of total protein from *P. boryanum*, cells were collected by centrifugation, resuspended in the sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (23), and lysed by heat treatment at 100°C for 3 min. SDS-PAGE was performed in the buffer system of Laemmli (23). Polypeptides were electrotransferred to a polyvinylidene membrane and allowed to react with the antibody against the NirA protein. A goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) was used as the second antibody to detect the reacting polypeptide.

Other methods. NiR activities were determined at 30°C, using toluene-permeabilized cells with dithionite-reduced methylviologen as the electron donor, as described by Herrero and Guerrero (19). Chlorophyll was determined as described by Mackinney (29), and protein was determined as described by Lowry et al. (26).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under accession number D31732.

RESULTS

Nucleotide and deduced amino acid sequences. Figure 2 shows the nucleotide sequence of a 2,700-bp fragment of DNA from *P. boryanum*, including the *nirA* gene for NiR (nucleotides 399 to 2363). The *nirA* gene encodes a protein of 654 amino acids with a calculated molecular weight of 72,135. Since the deduced NiR of the cyanobacterium *Synechococcus* sp. strain PCC7942 is composed of 512 amino acids (27, 42) and those from higher plants are composed of 561 to 565 amino acids excluding the transit peptide (5, 13, 21, 24), the deduced NiR of *P. boryanum* is by far the largest among the NiRs cloned from photosynthetic organisms. Comparison of the deduced amino acid sequence of the NirA protein of *P. boryanum* with those of NiRs from *Synechococcus* sp. strain PCC7942 and higher plants showed that the sequence from amino acids 1 to 511 of *Plectonema* NiR is similar to the other NiR sequences. The extent of identity is 62% with the NiR from *Synechococcus* sp. strain PCC7942 (Fig. 3) and 51 to 52% with NiRs from the higher plants (alignment not shown). The C-terminal portion of *Plectonema* NiR (amino acids 584 to 654), on the other hand, was found to be 30 to 35% identical to ferredoxins from cyanobacteria and higher plants, with all of the four Cys residues involved in binding of the [2Fe-2S] cluster in the plant-type ferredoxins being conserved (Fig. 3).

In the DNA region downstream of *nirA*, an open reading frame was found to start with an ATG codon located 97 bp from the termination codon of *nirA* (position 2460 in Fig. 2) and to extend beyond nucleotide 2700 (Fig. 2). Preliminary analysis of the nucleotide sequence farther downstream of

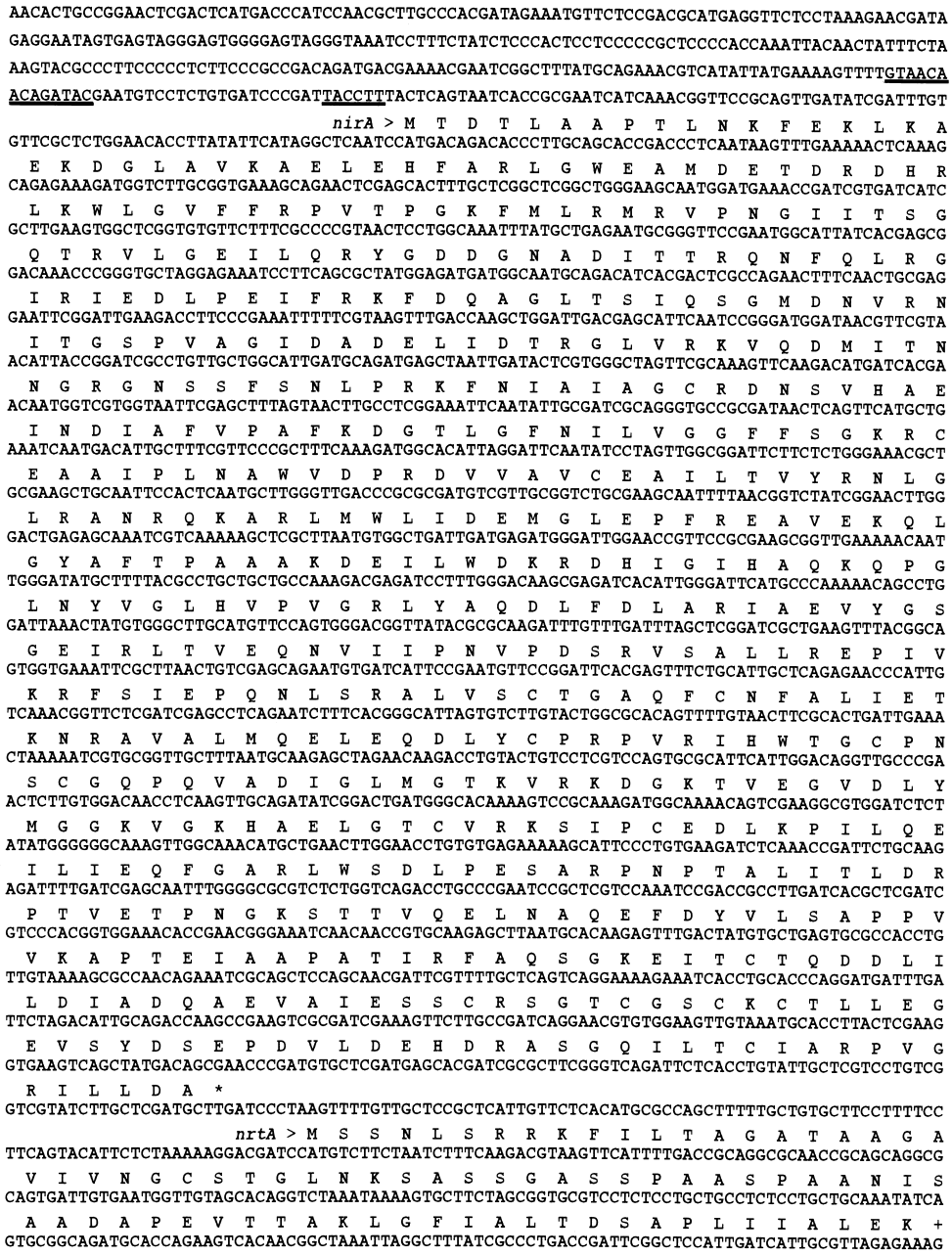

FIG. 2. Nucleotide sequence of a 2,700-bp fragment of DNA from *P. boryanum*, including the *nirA* gene for NiR (nucleotides 399 to 2363) and the 5′ portion of the putative *nrtA* gene (nucleotides 2460 to 2700). The deduced amino acid sequence of NiR and that of the putative NrtA protein are shown above the nucleotide sequence. The putative NtcA-binding site and the putative −10 promoter element are underlined.

nucleotide 2700 showed that the open reading frame is 1,335 nucleotides long, with the deduced amino acid sequence being 63% identical to that of *nrtA*, the gene located downstream of *nirA* and required for active transport of nitrate in *Synechococcus* sp. strain PCC7942 (29a, 33, 35). Thus, the open reading frame downstream of *Plectonema nirA* was tentatively identified as the *nrtA* gene of the cyanobacterium.

Transcription of *nirA* and its regulation. Northern hybridization analysis of total RNA from *P. boryanum* was performed, with an internal fragment of *nirA* as a probe, to determine the size and amount of the transcript (Fig. 4, lanes 1 and 2). There was no detectable amount of the transcript of *nirA* in RNA from the cells grown with ammonium (Fig. 4, lane 1). Transfer of the ammonium-grown cells to a nitrate-containing medium induced accumulation of the transcript (Fig. 4, lane 2). The hybridization signal was smeared, extending from 0.25 to >7 kb in terms of the size of the hybridizing fragments. Similar hybridization profiles were obtained with a fragment of the putative *nrtA* gene as a probe (Fig. 4, lanes 3 and 4). These findings were essentially the same as those obtained previously for *Synechococcus* sp. strain PCC7942 (42), in which *nirA* is the first gene of the *nirA-nrtABCD-narB* operon required for uptake and reduction of nitrate. The genes required for nitrate assimilation may form an operon in *P. boryanum* as well.

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P.b. NiR   1'  MTDTLAAPTLNKFEKLKAEKDGLAVKAELEHFARLGWEAMDETDRDHRLKWLGVFFRPVTPGKFMLRMRVPNGIITSGQTRVLGEILQRYGDDGNADIT
7942 NiR   1" MAQATATTEKLNKFEKLKLEKDGLAVRDQIQHFASIGWEAMDPGDREHRLKWLGIFWRPVTPGRFMARLRIPSGILQSQQLNALANFLQRYGDQASIDIT

100' TRQNFQLRGIRIEDLPEIFRKFDQAGLTSIQSGMDNVRNITGSPVAGIDADELIDTRGLVRKVQDMITNNGRGNSSFSNLPRKFNIAIAGCRDNSVHAEI
101" TRQNLQLRGLLLEDTPEFLERLHAVGLTSVQSGMDNVRNITGSPVAGLDAAELFDTRSLIQALQDDLTAAGQGNSEFTNLPRKFNIAIEGGRDNSIHAEI

200' NDIAFVPAFKDGTLGFNILVGGFFSGKRCEAAIPLNAWVDP-RDVVAVCEAILTVYRNLGLRANRQKARLMWLIDEMGLEPFREAVEKQLGYAFTPAAAK
201" NDLAFTPAYQDGTLGFNVWVGGFFSSTRVAPAIPLNAWVPADHSVIRLSRAILEVFRDNGSRGNRQKTRLMWLIDEWGIERFRQVVSEAYGAPLAAAAP-

299' DEILWDKRDHIGIHAQKQPGLNYVGLHVPVGRLYAQDLFDLARIAEVYGSGEIRLTVEQNVIIPNVPDSRVSALLREPIVKRFSIEPQNLSRALVSCTGA
300" ELMDWEKRDFLGVHPQKQAGLNFVGLHVPVGRLTTEDLYELARLADTYGQGEVRLTVEQNVILTHIPDAQLPTLLAEPLLTRFSPQPAPLSRGTVSCTGS

399' QFCNFALIETKNRAVALMQELEQDLYCPRPVRIHWTGCPNSCGQPQVADIGLMGTKVRKDGKTVEGVDLYMGGKVGKHAELGTCVRKSIPCEDLKPILQE
400" QYCNFALIETKQRAIAIAQSLEAELDLPRPVRIHWTGCPNSCGQPQVADIGLMGAKVRKDGQMVEGVDIFLGGKVGYDAHLGEKAMTGVACEDLPDVLRQ

499' ILIEQFGARLWSDLPESARPNPTALITLDRPTVETPNGKSTTVQELNAQEFDYVLSAPPVVKAPTEIAAPATIRFAQSGKEITCTQDDLILDIADQAEVA
500" LLIERFGAQARSH                                                    7002 Fd  1" MATYKVTLITPDGEVSYDAPDDEYILDSAGDAGYD

599' IESSCRSGTCGSCKCTLLEGEVSYDSEPDVLDEHDRASGQILTCIARPVGRILLDA
 36" LPASCRAGACSTCAGKIVSGTVD-QSEQSFLDDDQIEAGYVLTCIAYPQSDVTIETNKEEELY
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FIG. 3. Alignment of the deduced sequence of the NiR of *P. boryanum* (*P.b.* NiR) with those of the NiR of *Synechococcus* sp. strain PCC7942 (7942 NiR) (27, 42) and the ferredoxin of *Synechococcus* sp. strain PCC7002 (7002 Fd) (25) as optimized by the FASTA program (36). Vertical lines indicate aligned and identical amino acid residues. The dots indicate conservative replacements of amino acid residues. The four cysteine residues assumed to constitute binding sites for the [4Fe-4S] cluster and the siroheme are overlined and underlined, respectively, in the NiRs from *P. boryanum* and *Synechococcus* sp. strain PCC7942. The four cysteine residues involved in binding of [2Fe-2S] cluster in plant-type ferredoxins are doubly underlined in the ferredoxin of *Synechococcus* sp. strain PCC7002, and the corresponding cysteine residues in the C-terminal portion of *P. boryanum* NiR are doubly overlined.

As in *Synechococcus* sp. strain PCC7942 (42), the amount of the *nirA* transcript was largest at around 30 min after the transfer of ammonium-grown *Plectonema* cells to nitrate-containing medium (Fig. 5A) and then decreased gradually as the activity of NiR increased (Fig. 5B). Addition of ammonium to the cells, during accumulation of the *nirA* transcript, caused a rapid decline in the amount of the *nirA* transcript (Fig. 5A). Accumulation of the *nirA* transcript was observed also upon transfer of ammonium-grown cells to a medium containing no combined nitrogen, although the maximum amount of the transcript was smaller than that after the transfer to the nitrate-containing medium (Fig. 5A), and the development of NiR activity was marginal (Fig. 5B). These findings showed that the transcription of *nirA* is negatively regulated by ammo-

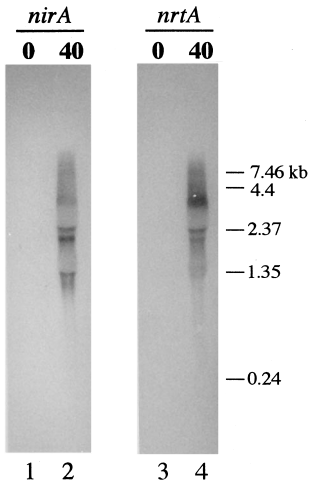

FIG. 4. Northern blot analysis of total RNA from *P. boryanum*. Cells were grown with ammonium as the nitrogen source and then transferred to nitrate-containing medium. RNA samples extracted before (lanes 1 and 3) and 40 min after (lanes 2 and 4) the transfer were compared. The RNA samples (10 μg per lane) were denatured, fractionated by electrophoresis, transferred to positively charged nylon membranes, and hybridized with gene-specific probes as indicated. The probes were detected by autoradiography (12 h exposure to X-ray film).

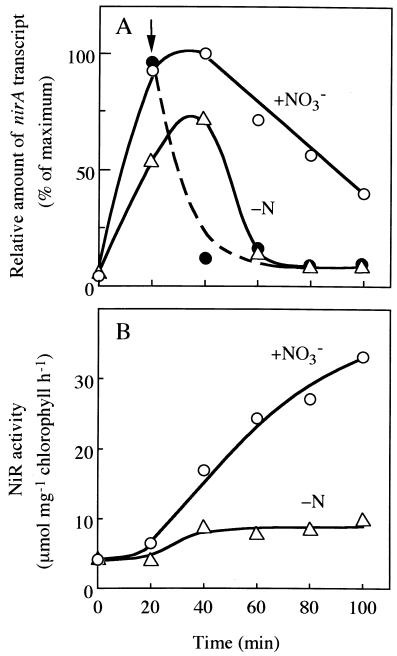

FIG. 5. Changes in levels of *nirA* transcript (A) and NiR activity (B) after transfer of ammonium-grown cells of *P. boryanum* to nitrate-containing medium (open circles) or to a medium containing no combined nitrogen (triangles). At the time indicated by the arrow in panel A, ammonium (7.5 mM) was added to a suspension of cells transferred to nitrate-containing medium, and the change in the amount of *nirA* transcript was monitored (closed circles and broken line).

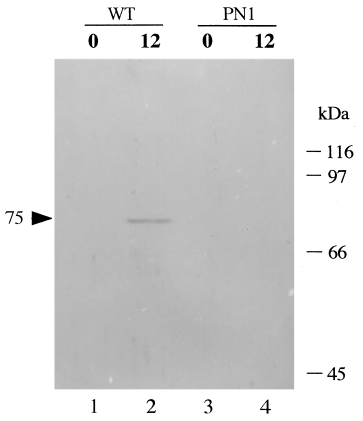

FIG. 6. Immunoblotting profiles of the proteins from the cells of the wild-type strain (WT; lanes 1 and 2) and the mutant PN1 (lanes 3 and 4), obtained by using the antiserum against the NiR polypeptide. Cells were grown with ammonium as the nitrogen source and then transferred to nitrate-containing medium. Samples extracted before (lanes 1 and 3) and 12 h after (lanes 2 and 4) the transfer were compared. Samples containing 30 μg of protein were electrophoresed in an SDS–8% polyacrylamide gel. After the electrophoresis, proteins in the gel were electrotransferred to a polyvinylidene difluoride membrane for immunostaining.

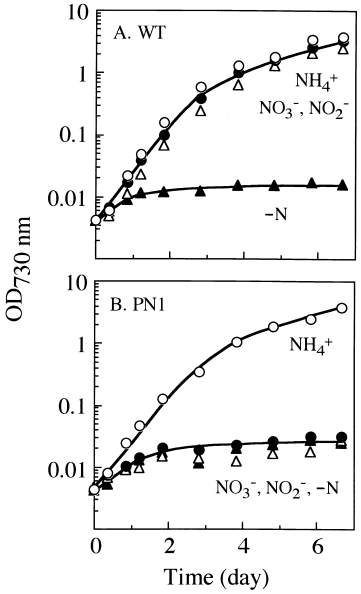

FIG. 7. Growth curves of the wild-type strain (A) and the mutant PN1 (B) after transfer of cells that had been grown in the presence of 2.5 mM $(NH_4)_2SO_4$ to media containing 2.5 mM $(NH_4)_2SO_4$ (open circles), 5 mM $NaNO_2$ (open triangles), 5 mM KNO_3 (closed circles), and no combined nitrogen (closed triangles). OD_{730} nm, optical density at 730 nm.

nium in *P. boryanum* as in *Synechococcus* sp. strain PCC7942 (42) and is derepressed upon deprivation of ammonium from the medium. Luque et al. have shown that in *Synechococcus* sp. PCC7942, ammonium-repressible genes have a promoter with the consensus sequence $GTAN_8TACN_{22}TAN_3T$, where $GTAN_8TAC$ is the binding site for NtcA, the global nitrogen regulator protein, and TAN_3T is the −10 promoter sequence (28). In the region upstream of *nirA* of *P. boryanum*, the nucleotide sequence from positions −134 to −94 with respect to the *nirA* coding region (nucleotides 265 to 306 in Fig. 2) exactly matches the proposed consensus sequence of the ammonium-repressible promoters and was tentatively identified as the promoter of the *nirA* gene of *P. boryanum.*

Identification of the NiR polypeptide. Immunoblotting analysis of total protein of wild-type cells of *P. boryanum* was performed, using the antibody against the NirA polypeptide expressed in *E. coli* (Fig. 6, lanes 1 and 2). While no protein band was stained in the extract of ammonium-grown cells (Fig. 6, lane 1), a polypeptide was stained in the extract of the cells that had been incubated in the nitrate-containing medium for 12 h (Fig. 6, lane 2). The apparent molecular mass of the stained polypeptide was 75 kDa, which was close to the calculated molecular mass of the NirA polypeptide. On the basis of its reactivity with the antibody, its apparent molecular mass, and its absence in ammonium-grown cells, the 75-kDa polypeptide was identified as the product of the *nirA* gene.

Characterization of an insertional mutant of *nirA*. A defined mutant, PN1, was constructed by inserting a kanamycin resistance gene into the *Eco*RV site in the *nirA* coding region (Fig. 1), and its phenotype was examined. To confirm the gene interruption, the genomic DNA from the mutant was analyzed by the PCR method with a pair of *nirA*-specific primers that amplifies a 200-bp *nirA* fragment of wild-type genome carrying the *Eco*RV site. The PCR on the genomic DNA from PN1 amplified a 1.5-kbp DNA fragment, which confirmed the insertion of a 1.3-kbp kanamycin resistance gene into *nirA* (data not shown). In accordance with these results, immunoblotting analysis of the extract of PN1 showed that the 75-kDa polypeptide representing the NirA protein (Fig. 6, lane 2) was missing in the mutant (Fig. 6, lanes 3 and 4).

Figure 7 compares the growth curves of the wild-type strain and the insertional mutant PN1. The wild-type strain grew well with nitrate, nitrite, and ammonium. PN1 grew as fast as the wild-type strain in a medium containing ammonium but failed to grow in a medium containing nitrate or nitrite as the nitrogen source. PN1 showed no NiR activity not only when grown with ammonium but also after a 12-h incubation of the cells in a nitrate-containing medium. These findings demonstrated that *nirA* is the sole NiR gene of *P. boryanum.*

DISCUSSION

The deduced NirA polypeptide of *P. boryanum* is a fusion of NiR and ferredoxin-like domains (Fig. 3), having a calculated molecular mass of 72 kDa. The molecular mass of the polypeptide in the extract of *P. boryanum* cells is estimated by SDS-PAGE and immunoblotting to be 75 kDa (Fig. 6), which is close to the predicted molecular mass. This finding indicates that NiR of *P. boryanum* has the C-terminal extension in vivo. The absence of NiR activity in the insertional mutant of *nirA* shows that the novel protein is the sole NiR species in the cyanobacterium.

Structural genes for ferredoxin-dependent NiR have been cloned from various species of higher plants and shown to encode polypeptides of 561 to 565 amino acids (molecular mass = 63 kDa) excluding the transit peptide (5, 13, 21, 24). The deduced NiR polypeptide of the cyanobacterium *Synechococcus* sp. strain PCC7942 is smaller than those from higher plants, consisting of 512 amino acid residues (molecular mass = 56.5 kDa), and is homologous to the higher plant enzyme (27, 42). The NiR preparations from other strains of cyanobacteria have apparent molecular masses of 54 to 58 kDa as determined by SDS-PAGE (4, 30, 31), suggesting that they consist only of the NiR domain conserved in cyanobacterial and higher plant enzymes. The occurrence of the C-terminal

extension to the NiR domain is therefore an unusual property of the NiR of *P. boryanum*.

The function of the C-terminal domain of *Plectonema* NiR is currently unknown. Occurrence of a domain similar to the [2Fe-2S] ferredoxin has been reported in phthalate dioxygenase reductase of *Pseudomonas cepacia*, in which the ferredoxin-like domain is involved in the intramolecular electron transport reaction (9). Since NiRs of other cyanobacteria and higher plants receive electrons from ferredoxin, the ferredoxin-like domain of *Plectonema* NiR may function as an electron donor to the NiR portion of the same molecule. Biochemical studies of the purified NiR from *P. boryanum* are required to determine (i) whether the ferredoxin-like domain binds a [2Fe-2S] cluster and (ii) whether the ferredoxin-like domain can donate electrons to the NiR domain.

Ammonium plays a major role in the regulation of nitrate assimilation in the cyanobacterium *Synechococcus* sp. strain PCC7942 by repressing the relevant genes (27, 42). This seems to be true also in *P. boryanum*, since transcription of *nirA* is repressed by ammonium (Fig. 5A). As in *Synechococcus* sp. strain PCC7942, transcription of *nirA* in *P. boryanum* is derepressed simply by removal of ammonium from the medium and does not require the presence of nitrate (Fig. 5A). The development of NiR activity, on the other hand, is strongly stimulated by the presence of nitrate (Fig. 5B), which is in contrast to the case for *Synechococcus* sp. strain PCC6301 (also known as *Anacystis nidulans*), a strain closely related to strain PCC7942, in which nitrate is not strictly required for the development of NiR activity (19). Nitrate has been suggested to act as an inducer of the development of NR activity in nitrogen-fixing strains but not in non-nitrogen-fixing strains of cyanobacteria (17, 18). Since *P. boryanum* is a nitrogen fixer and *Synechococcus* sp. strain PCC6301 is not, nitrate may also act as an inducer of the development of NiR activity specifically in nitrogen-fixing strains.

The actual function of nitrate in the induction of NiR activity in *P. boryanum* is currently unknown. As has been discussed above, nitrate is clearly not required for the derepression of *nirA*, the structural gene for NiR (Fig. 5A). The steps of de novo NiR biosynthesis that could require nitrate include post-transcriptional and translational regulation of *nirA* expression. The biosynthesis of the cofactor of NiR, siroheme, and its assembly to the apoprotein might also be dependent on nitrate. Further studies are required for the elucidation of the molecular basis of the nitrate requirement in the development of NiR activity in *P. boryanum.*

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