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Identification and Characterization of Two Nitrogen-Regulated Genes of the Cyanobacterium *Synechococcus* sp. Strain PCC7942 Required for Maximum Efficiency of Nitrogen Assimilation

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Two nitrogen-regulated genes were found in the genomic DNA region upstream of the *nirA* operon involved in uptake and utilization of nitrate in *Synechococcus* sp. strain PCC7942. The two genes (*nirB* and *ntcB*) are transcribed divergently from *nirA* and encode proteins of 349 and 309 amino acid residues, respectively. The levels of *nirB* and *ntcB* transcripts were low in cells growing on ammonium and increased upon transfer of ammonium-grown cells to nitrate-containing medium. The deduced NirB protein sequence has no similarities to other known proteins, whereas the deduced NtcB protein sequence is homologous to bacterial transcriptional activators of the LysR family. Defined mutants constructed by interrupting *nirB* or *ntcB* with a drug resistance marker grew as fast as the wild-type strain on ammonium but grew slower than the wild-type strain on nitrate or nitrite. The *nirB* mutant had higher activities of nitrate reductase, glutamine synthetase, and glutamate synthase than the wild-type strain, but its nitrite reductase activity was 40% of the wild-type levels. The mutant excreted nitrite into the medium during growth on nitrate, showing that nitrite reductase limits nitrate assimilation. These findings suggested that *nirB* is required for expression of maximum nitrite reductase activity. When grown on ammonium, the *nirB* mutant grew normally but cultures of the *ntcB* mutant still showed a yellowish-green color typical of nitrogen-limited cells. NtcB seems to regulate utilization of fixed nitrogen by controlling the expression of a certain gene(s) involved in nitrogen metabolism.

Nitrate is the major source of nitrogen for cyanobacteria (12, 13). It is transported into the cells by an active transport system that belongs to the group of ABC transporters (28). Nitrate taken up into the cells is reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR), and the resulting ammonium is combined with glutamate by glutamine synthetase (GS) to form glutamine. The amide nitrogen of glutamine is further transferred to other compounds by glutamine amidotransferases, including glutamate synthase (GOGAT), to form various organic nitrogenous compounds. While cyanobacterial NR, a ferredoxin-dependent enzyme, is distinct from the pyridine nucleotide-dependent NRs of higher plants (3, 17), the ferredoxin-dependent NiR of cyanobacteria is highly homologous to that of higher plants (22, 36).

In the unicellular cyanobacterium *Synechococcus* sp. strain PCC7942, the genes encoding the nitrate transporter (*nrtABCD*) (27–29), NR (*narB*) (3, 18), and NiR (*nirA*) (22, 36) form an operon *nirA-nrtABCD-narB* (36), and transcription of the operon is negatively regulated by ammonium either added to the medium or generated internally by reduction of nitrate (36). Since inhibition of GS or glutamine amidotransferases results in activation of transcription of the *nirA* operon, we have concluded that accumulation of a compound synthesized by transfer of the amide nitrogen from glutamine, or a derivative of this compound acts as a regulatory signal for repression of the *nirA* operon (36). On the other hand, a Crp-type DNA-binding protein, NtcA (37), has been shown to be required for activation of the *nirA* operon and to have binding sites in the promoter region of *nirA* (23). This protein activates transcription of the genes for GS (*glnA*) and itself (*ntcA*) as well and hence is a global nitrogen regulator of the cyanobacterium (23, 37).

In addition to the well-characterized genes mentioned above, there certainly are more genes related to the expression and regulation of nitrate-assimilating activity, including those required for biosynthesis and assembly of the cofactors of NR and NiR. In *Synechococcus* sp. strain PCC7942, two genes required for expression of NR activity, *narA* and *narC*, have been cloned, although their functions are uncertain (18, 19). Elucidation of the functions of these genes and identification and characterization of other genes related to nitrate utilization would be necessary for a comprehensive understanding of the nitrate-assimilating mechanism and its regulation. In this communication, we present a characterization of the region of DNA upstream of *nirA* and show the presence of two nitrogen-regulated genes (designated *nirB* and *ntcB*). From the phenotype of a mutant strain constructed by inactivation of *nirB*, it is deduced that the gene is required for expression of maximum NiR activity. A targeted *ntcB* mutant, on the other hand, shows a discernible lesion not only when grown on nitrate or nitrite but also when grown on ammonium. On the basis of the deduced primary structure of NtcB, the possible role of this protein in the regulation of nitrogen metabolism is discussed.

MATERIALS AND METHODS

Strains and growth conditions. Cells of *Synechococcus* sp. strain PCC7942 were grown photoautotrophically under continuous illumination provided by fluorescent lamps (70 microeinsteins m^{-2} s^{-1}). The growth temperature was 30°C unless otherwise stated. The basal medium used was a nitrogen-free medium obtained by replacing $NaNO_3$, $Co(NO_3)_2$, and ferric ammonium citrate in medium BG11 (34) with NaCl, $CoCl_2$, and ferric citrate, respectively. Media containing various nitrogen sources were obtained by adding 3.75 mM $(NH_4)_2SO_4$, 15 mM KNO_3, or 5 mM $NaNO_2$ to the basal medium. 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*

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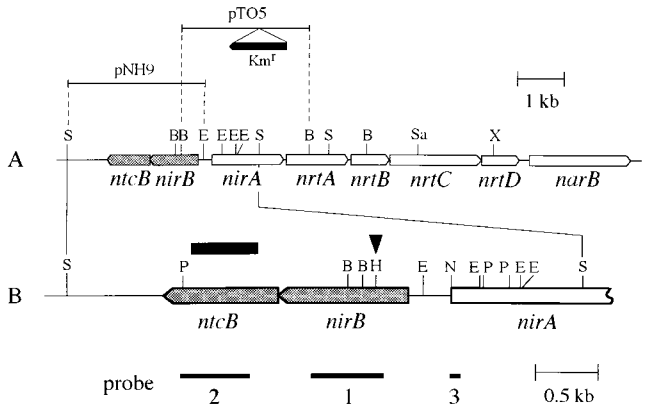

FIG. 1. *nirB* and *ntcB* genes in the region upstream of the *nirA* operon. (A) Restriction map of the *ntcB-narB* region of the *Synechococcus* sp. strain PCC7942 genome that is regulated by nitrogen availability. Previously identified genes are shown as open bars denoting the direction of transcription. The genes identified in the present study are shown as stippled bars. Above the map, the fragments of genomic DNA cloned into pTO5 and pNH9 are shown. (B) Restriction map of the *nirA*, *nirB*, and *ntcB* genes. The thick bars below the map show the probes used for Northern hybridization analysis and dot blot analysis (probes 1 to 3). Above the map, the site where an insertion into the *nirB* mutant was made is shown by a triangle and the genome region deleted in the *ntcB* mutant is shown by a rectangle. Restriction endonuclease sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I; X, *Xba*I.

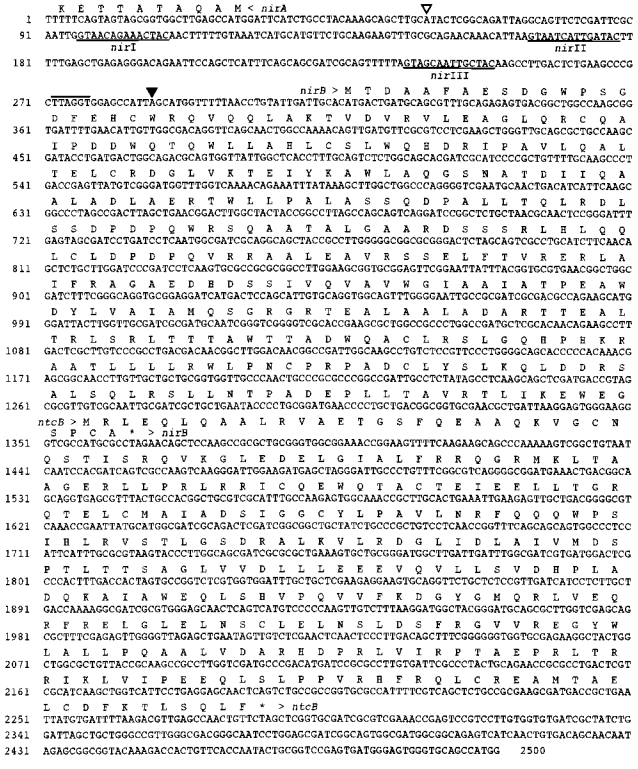

FIG. 2. Nucleotide sequence of a 2,500-bp fragment of DNA from *Synechococcus* sp. strain PCC7942, including the *nirB* and *ntcB* genes, as well as the 5′ region of *nirA*. The deduced amino acid sequences of NirB, NtcB, and NirA are shown above the nucleotide sequence. The site of initiation of *nirB* transcription is indicated by a closed arrowhead, while that of *nirA* transcription (36) is indicated by an open arrowhead. NtcA-binding motifs *nir*I, *nir*II, and *nir*III (23) are underlined, and the putative −10 promoter element of *nirB* is overlined.

coli DH5α (Bethesda Research Laboratories) and JM105, used as hosts for plasmid constructions and M13 vectors, respectively, were grown on Luria-Bertani medium supplemented with ampicillin (50 μg/ml) and/or kanamycin (50 μg/ml) when appropriate.

Cloning and DNA sequence determination. Cloning of a fragment of *Synechococcus* DNA carrying the *nirA* gene with a 1.3-kbp kanamycin resistance (Km^r) gene inserted at the internal *Sac*I site (pTO5; Fig. 1) was described previously (36). A 0.5-kbp *Bam*HI-*Eco*RI fragment of pTO5, which contains the 5′-flanking sequence of *nirA*, was used as a probe for cloning of the DNA region farther upstream from *nirA*. A 3.0-kbp *Sac*I-*Eco*RI fragment of *Synechococcus* DNA was isolated by colony hybridization from a genomic library of *Synechococcus* sp. strain PCC7942 that was constructed by ligating an *Eco*RI-*Sac*I digest of chromosomal DNA into pUC19 (pNH9; Fig. 1). For nucleotide sequence analysis, the 3.0-kbp *Sac*I-*Eco*RI fragment was cloned into M13 mp18/19 and nested deletions were generated by using an exonuclease III-mung bean nuclease system (31). Single-stranded DNA templates were prepared as described by Vieira and Messing (39) and sequenced by the dideoxy-chain termination method (32) by using fluorescent primers or [α-^{32}P]dCTP for chain detection. Both DNA strands were sequenced completely with no ambiguities. Database homology searches were performed with the BLAST program (2). A search for potential helix-turn-helix structures in the deduced amino acid sequences was performed by the method of Dodd and Egan (8).

Insertional inactivations. For insertional mutagenesis of *nirB*, pNH9 was partially digested with *Hin*dIII and Km^r cartridge C.K1 (9), from Tn*5* (4), was inserted into the *Hin*dIII site in *nirB*. For mutagenesis of *ntcB*, the insert from a deletion clone of pNH9, containing the 5′ region of the gene and the flanking sequence (nucleotides 585 to 1553 in Fig. 2), was excised and cloned into another deletion clone containing the 3′ region of the gene and the flanking sequence (from nucleotide 2090 in Fig. 2 to the *Sac*I site distal to *ntcB*), so that the two inserts are tandemly joined in a plasmid, with a *Kpn*I site at their junction. A Km^r cartridge from Tn*903* (26) was excised from pUC4K (38) and inserted at the junction of the two portions of *ntcB*. The resulting plasmid contained a modified *ntcB* gene in which a 536-bp internal segment (nucleotides 1554 to 2089 in Fig. 2) had been replaced by the drug resistance gene. For construction of an insertion mutant of the *ntcA* gene (37), a 496-bp internal fragment of the gene, carrying nucleotides +82 to +577 with respect to the translation start site, was amplified by PCR and digested with *Bam*HI and *Hin*dIII. The resulting 403-bp *Bam*HI-*Hin*dIII fragment of *ntcA* was cloned into pUC19, and the Km^r cartridge excised from pUC4K was inserted between the two *Pst*I sites in the cloned *ntcA* fragment. In each of the constructions, the Km^r-encoding gene was inserted in the same orientation as the gene being interrupted. The resulting plasmids were used to transform *Synechococcus* sp. strain PCC7942 to kanamycin resistance through homologous recombination in accordance with the protocol reported by Williams and Szalay (42). The transformants were allowed to grow on solid medium supplemented with 10 μg of kanamycin per ml and 3.75 mM $(NH_4)_2SO_4$. After three serial streak purifications to segregate homozygous mutants (41), genomic DNA was isolated and analyzed by PCR to confirm the presence and position of the Km^r gene.

DNA isolation and analysis by PCR. Chromosomal DNAs were extracted and purified from the wild-type and mutant *Synechococcus* cells as described by Williams (41). The PCR was carried out on approximately 30 ng of chromosomal DNA by using two pairs of primers specific for *nirB* and *ntcB*, respectively. The oligonucleotides used as primers were synthesized in accordance with the sequence shown in Fig. 2: the sequence from bases 450 to 466 and the complementary sequence from bases 794 to 810 for *nirB*; the sequence from bases 1530 to 1549 and the complementary sequence from bases 2124 to 2142 for *ntcB*.

RNA isolation and analysis. Total RNA was extracted and purified from *Synechococcus* cells by the method of Aiba et al. (1). RNA samples 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). The blots were allowed to hybridize with probes specific to each of the *nirA*, *nirB*, and *ntcB* genes as described by Church and Gilbert (6). The following gene-specific probes were used: a 530-bp fragment of *nirB* (probe 1 in Fig. 1), a 510-bp fragment of *ntcB* (probe 2 in Fig. 1), and a 20-mer oligonucleotide (GTAGCGGTGGCTTGAGCCAT) complementary to nucleotides +1 to +20 of the coding region of the *nirA* gene (probe 3 in Fig. 1). The double-stranded DNA probes were labeled with ^{32}P as described by Feinberg and Vogelstein (10). [γ-^{32}P]ATP and T4 polynucleotide kinase were used to label the oligonucleotide probe. The hybridization signals were detected by autoradiography on X-ray film or by a Bio-image analyzer (Fuji Photo Film). Changes in the levels of *nirB* and *ntcB* transcripts were monitored by dot blot analysis with the gene-specific probes described above. The radioactivity of the RNA dots was quantified with a Bio-image analyzer.

For mapping of the 5′ end of the transcript from the *nirB* gene by S1 nuclease protection experiments, a 0.9-kbp *Bam*HI-*Pst*I fragment of pTO5 (36), carrying the 5′ region of *nirB* and the flanking sequence, was subcloned into pUC19. The plasmid was cut by *Nco*I at position −290 with respect to the *nirB* open reading frame (ORF; at position 28 in Fig. 2). Single-stranded DNA was generated by single-primer PCR with the *Nco*I-cleaved plasmid as the template and a 19-mer oligonucleotide complementary to nucleotides +2 to +20 of the *nirB* coding region (nucleotides 318 to 336 in Fig. 2) as the primer. The single-stranded DNA was purified by electrophoresis on a 1.5% agarose gel and labeled by use of

[γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled single-stranded DNA was allowed to hybridize with 40 μg of RNA from *Synechococcus* sp. strain PCC7942 and then treated with S1 nuclease in accordance with the standard protocol (31). The size of the DNA fragment protected from digestion by S1 nuclease was determined by electrophoresis on a 6% polyacrylamide gel. A sequence ladder generated with the plasmid carrying the 0.9-kbp *Bam*HI-*Pst*I fragment as the template and the 19-mer oligonucleotide as the primer was the source of the size markers used.

Enzyme assays. NR and NiR activities were determined by using toluene-permeabilized cells with dithionite-reduced methylviologen as the electron donor in accordance with the methods of Herrero et al. (15) and Herrero and Guerrero (16), respectively. For determination of GS and GOGAT activities, cell extracts were prepared as described by Marqués et al. (25). Transferase activity of GS was determined as described by Cullimore and Sims (7), and GOGAT activity was determined as described by Marqués et al. (25), except that dithionite-reduced methylviologen was used in place of reduced ferredoxin as the electron donor (20). Enzyme activities were assayed at 30°C.

Determination of nitrite in the medium. Cells of *Synechococcus* sp. strain PCC7942 were grown in an ammonium-containing medium and harvested at the mid-logarithmic phase of growth by centrifugation at 3,500 × *g* for 5 min at 25°C. The collected cells were washed with nitrogen-free medium by resuspension and recentrifugation and inoculated into fresh nitrate-containing medium to a concentration of 6 μg of chlorophyll per ml. Aliquots of 1 ml were withdrawn from the cultures at regular time intervals. After removal of the cells by passage through a 0.22-μm-pore-size cellulose acetate filter (Toyo Roshi), nitrite in the medium was determined by the method of Snell and Snell (33).

Analytical methods. Chlorophyll was determined as described by Mackinney (24), and protein was determined as described by Lowry et al. (21).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D16303.

RESULTS

Nucleotide and deduced amino acid sequences. In a previous study (36), we cloned a DNA fragment that carries *nirA* with a Km^r gene inserted at the internal *Sac*I site (pTO5; Fig. 1). The fragment carried 663 bp of the 5′ flanking sequence of *nirA*, as shown by nucleotide sequence analysis. With a 0.5-kbp *Bam*HI-*Eco*RI fragment of pTO5 as the probe, a 3.0-kbp *Sac*I-*Hin*dIII fragment that carries the region farther upstream from *nirA* was cloned (pNH9; Fig. 1) and its sequence was determined. Figure 2 shows the nucleotide sequence of a 2,500-bp fragment of *Synechococcus* DNA carrying the region upstream of *nirA*. Two long ORFs, which are oriented divergently from *nirA*, were identified within the region (designated *nirB* and *ntcB*; Fig. 1 and 2). The two ORFs overlap each other by seven bases and would encode proteins of 349 and 309 amino acid residues, respectively.

The deduced sequence of hydrophilic protein NirB has no significant similarities to known protein sequences. The deduced NtcB protein sequence, on the other hand, shows a similarity extending over more than 280 amino acid residues to the members of the LysR family of bacterial transcriptional activators (14), such as GltC of *Bacillus subtilis* (5) and IlvY (40) and LysR (35) of *E. coli*. It is 27% identical to GltC (Fig. 3) and 24% identical to IlvY and LysR. Typical of the LysR family, the greatest similarities are near the N termini of these proteins. The first 60 amino acid residues of NtcB are 46, 41, and 33% identical to GltC, IlvY, and LysR, respectively. Like other members of the LysR family, NtcB has a putative helix-turn-helix DNA-binding motif in its N-terminal region, from amino acids 18 to 37 (overlined in Fig. 3). For this amino acid segment, a score for evaluation of potential helix-turn-helix motifs was calculated as described by Dodd and Egan (8) to be 2269. This corresponds to a standard deviation score of 6.9, which is higher than those of most of known helix-turn-helix motifs (8). These findings strongly suggest that NtcB is a DNA-binding protein of the LysR family with a helix-turn-helix structure near its N terminus.

Transcription of *nirB* and *ntcB* and their regulation. Northern (RNA) hybridization analysis of total RNA from *Synechococcus* sp. strain PCC7942 was performed with internal fragments of *nirB* and *ntcB* as probes to determine the size and level of the transcript (Fig. 4). The *nirB* and *ntcB* transcript level was low in ammonium-grown cells (Fig. 4, lanes 1 and 3). Transfer of ammonium-grown cells to a nitrate-containing medium induced transcript accumulation (Fig. 4, lanes 2 and 4). The hybridization signal was smeared, extending from 0.25 to 1.5 kb in terms of the size of the hybridizing fragments. The smeared appearance of the hybridization signals was not attributable to breakdown of RNA during extraction and analysis, since discrete hybridization signals due to *psbA* transcripts were obtained, as reported by Golden et al. (11), with the same preparation of RNA (data not shown). These results suggested that the transcripts of the two genes are rapidly degraded in vivo. Similar to the case of *nirA* (36), the levels of both *nirB* and *ntcB* transcripts in the wild-type strain were highest at around

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NtcB   1'        MRLEQLQAALRVAETGSFQEAAQKVGCNQSTISRQVKGLEDELGIALFRRQGR-
                   | ||.  .|||  .  |||...  .||.|||| .||.||...|| |.||
GltC   1" MDVRQTWSLRQLRYFMEVAEREHVSEAADHLHVAQSAISRQIANLEEELNVTLFEREGRN

       54' MKLTAAGERLLPRLRRICQEWQTACTEIEELLTGRQTELCMAIADSIGGCYLPAVLNRFQ
           .|||. |. .| ...   .. . | ..|.| |. ..... .....|...  ||.|.. |.
       61" IKLTPIGKEFLIHVKTAMKAIDYAKEQIDEYLDPHRGTVKIGFPTSLASQLLPTVISAFK

      114' QQWPSIHLRVSTLGSDRALKVLRDGLIDLAIVMDSPTLTTSAGLVVDLLLEEEVQVLLSV
           ..| ... .. . . ....|. |||..   || . .... ..|..|.. .|...
      121" EEYPHVEFLLRQGSYKFLIEAVRNRDIDLALLGPVPT--NFSDITGKILFTEKIYALVPL

      174' DHPLADQKAIAWEQLSHVPQVVFKDGYGMQRLVEQRFRELGLELNSCLELNSLDSFRGVV
           .||||.||..  .|.. . |.| .|. .. .. .  .. |..   . | ..||...|.|
      179" NHPLAKQKTVHLIDLRNDQFVLFPEGFVLREMAIDTCKQAGFAPLVSTEGEDLDAIKGLV

      234' REGYWLALLPQAALVDARHDPRLVIR-PTAEPRLTRRIKLVIPEEQLSLPPVRHFRQLCR
           ..|  ..||..... .||.... |.. |...| . .. |...   |....|
      239" SAGMGVTLLPESTFAET--TPRFTVKIPIEFPQVKRTVGIIKPKNRELAPSANDFYEFVI

      293' EAMTAELCDFKTLSQLF

      297" QFFSKLEQYQ
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FIG. 3. Alignment of the deduced amino acid sequence of NtcB with that of GltC of *B. subtilis* as optimized by the FASTA program (30). Vertical lines indicate aligned and identical amino acid residues. Dots indicate conservative replacements of amino acid residues. The putative helix-turn-helix structure is overlined in NtcB and underlined in GltC.

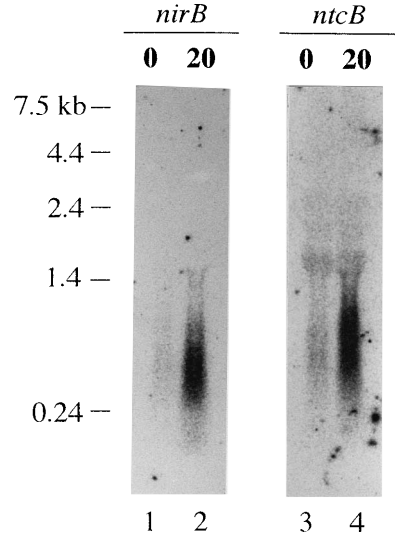

FIG. 4. Northern blot analysis of total RNA from wild-type *Synechococcus* sp. strain PCC7942. Cells were grown with ammonium as the nitrogen source and then transferred to nitrate-containing medium. RNA samples extracted before (designated 0; lanes 1 and 3) and 20 min after (designated 20; lanes 2 and 4) the transfer were compared. The RNA samples (20 μg per lane) were denatured, fractionated by electrophoresis, transferred to positively charged nylon membranes, and hybridized with gene-specific probes as indicated.

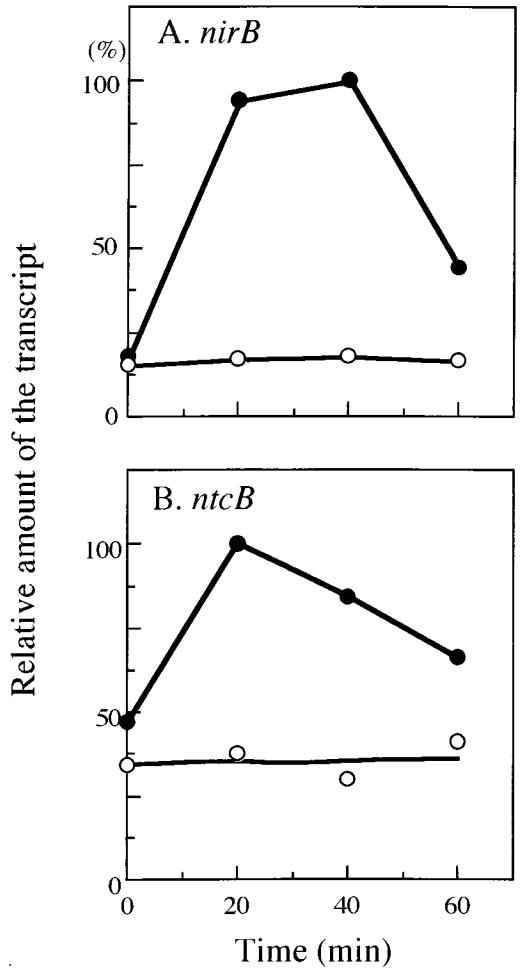

FIG. 5. Changes in *nirB* (A) and *ntcB* (B) transcript levels after transfer of ammonium-grown cells of the wild-type strain (closed circles) and an *ntcA* insertion mutant (open circles) to nitrate-containing medium.

30 min after the transfer of ammonium-grown cells to nitrate-containing medium and then decreased gradually (Fig. 5). Activation of transcription of the genes involved in nitrogen assimilation in *Synechococcus* sp. strain PCC7942, including *nirA*, has been shown to be dependent on *ntcA*, a gene encoding a Crp-type transcriptional activator (23, 37). In a targeted *ntcA* mutant, there was no activation of transcription of *nirB* and *ntcB* after transfer of ammonium-grown cells to nitrate-containing medium (Fig. 5). This showed that activation of *nirB* and *ntcB* is also dependent on NtcA. From the results of an S1 nuclease protection experiment (data not shown), the site of initiation of transcription of *nirB* was mapped to a position 30 nucleotides upstream from the initiation codon of *nirB* (nucleotide 287 in Fig. 2).

Gene inactivations. The activation of transcription of *nirB* and *ntcB* on transfer from ammonium-containing medium to nitrate-containing medium in an *ntcA*-dependent manner suggested that the genes are involved in the assimilation of nitrogen. To investigate the function of *nirB* and *ntcB*, defined mutants were constructed by inserting a Km^r cassette into each of the genes and their phenotypes were examined. To confirm the gene interruptions, the genomic DNAs from the mutants were analyzed by the PCR method with pairs of primers specific to each of genes *nirB* and *ntcB*. In both cases, the sizes of the PCR products changed in the way expected for a double homologous recombination event that results in interruption of the genes (data not shown).

TABLE 1. Doubling time and pigmentation of the wild-type and mutant strains on different nitrogen sources[a]

Mutation	Doubling time[b] (pigmentation)[c]		
	Potassium nitrate	Sodium nitrite	Ammonium sulfate
None (wild type)	4.7 (BG)	5.3 (BG)	4.4 (BG)
nirB	6.3 (YG)	7.1 (YG)	4.5 (BG)
ntcB	7.2 (YG)	7.8 (YG)	4.6 (YG)

[a] Wild-type *Synechococcus* sp. strain PCC7942 and mutant strains were grown at 35°C on nitrogen-deficient BG11 supplemented with 15 mM potassium nitrate, 5 mM sodium nitrite, or 3.75 mM ammonium sulfate and assayed for growth.
[b] Doubling time in hours was determined from the rise in optical density at 730 nm.
[c] Pigmentation of the cultures. Abbreviations: BG, cells grew without visible loss of pigmentation, being blue-green; YG, cells grew with moderate loss of pigmentation, being yellowish green.

Phenotype of the *nirB* mutant. Wild-type cells of *Synechococcus* sp. strain PCC7942 grew well with nitrate, nitrite, or ammonium as the sole source of nitrogen, being blue-green because of abundance of the major light-harvesting pigment protein phycocyanin. The *nirB* insertion mutant grew as fast as the wild-type strain with normal pigmentation in ammonium-containing medium but grew slower than the wild-type strain when nitrate or nitrite was the nitrogen source (Table 1). In nitrate- or nitrite-containing medium, the color of the mutant cells was yellowish green because of reduced pigmentation (Table 1), which is typical of the cultures under nitrogen limitation (29). These findings suggest that *nirB* is required for efficient utilization of nitrate and nitrite.

In *Synechococcus* sp. strain PCC7942, six genes involved in the utilization of nitrate and nitrite are clustered and transcribed as an operon (*nirA-nrtABCD-narB*) (28, 36). To determine whether the interruption of *nirB* had affected the transcription of the operon, Northern hybridization analysis was performed with total RNAs from the wild type and the mutant by using a probe specific to *nirA*, the first gene of the operon (Fig. 6, lanes 1 to 4). As previously described (36), transfer of ammonium-grown cells of the wild-type strain to nitrate-containing medium induced accumulation of the *nirA* operon transcript, whose size ranged from 0.2 to 5 kb (Fig. 6, lanes 1 and 2). The *nirB* mutant also accumulated the transcript of the operon when transferred to nitrate-containing medium (Fig. 6, lanes 3 and 4), and the level of the transcript was comparable to that in the wild-type strain. Thus, the lesion in the utilization of nitrate and nitrite in the *nirB* mutant was not at the step of transcription of the *nirA* operon.

Measurements of activities of the enzymes related to nitrogen assimilation showed that NR, GS, and GOGAT activities were higher in the *nirB* mutant than in the wild-type strain, whereas the activity of NiR in the *nirB* mutant was about 40% of that in the wild-type strain (Table 2). During growth with nitrate as the nitrogen source, the *nirB* mutant excreted nitrite into the medium (Fig. 7), which showed that the step of nitrite reduction limits nitrate assimilation in the mutant. Under the same growth conditions, the wild-type strain and the *ntcB* mutant did not excrete nitrite into the medium (Fig. 7). These findings suggested that *nirB* is required for expression of maximum NiR activity.

Phenotype of the *ntcB* mutant. Cells of the *ntcB* mutant grew slower than those of the wild-type strain and were yellowish

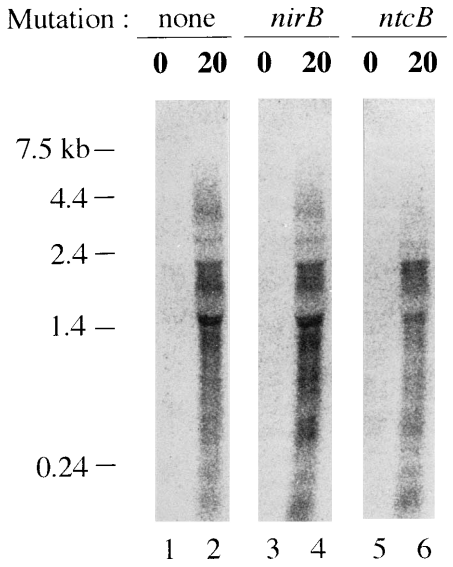

FIG. 6. Northern blot analysis of total RNAs from the wild-type strain (lanes 1 and 2) and the *nirB* (lanes 3 and 4) and *ntcB* (lanes 5 and 6) mutants using a probe specific for *nirA*. Cells were grown with ammonium as the nitrogen source and then transferred to nitrate-containing medium. RNA samples extracted before (designated 0; lanes 1, 3, and 5) and 20 min after (designated 20; lanes 2, 4, and 6) 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 the gene-specific probe.

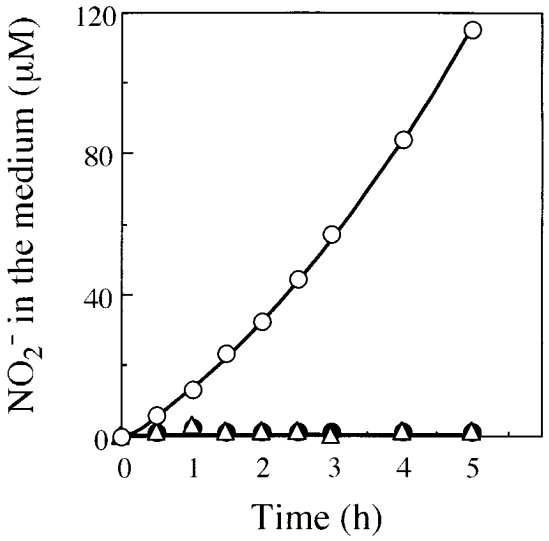

FIG. 7. Changes in nitrite concentration in the medium of the wild-type strain (closed circles) and the *nirB* (open circles) and *ntcB* (open triangles) mutants after transfer of ammonium-grown cells to nitrate-containing medium. Cells were harvested, washed, and inoculated into nitrate-containing medium at time zero to a concentration of 6 μg of chlorophyll per ml.

green when nitrate or nitrite was the nitrogen source (Table 1). In ammonium-containing medium, the *ntcB* mutant grew as fast as the wild-type strain but the cells were yellowish green. These results showed that the gene is required for normal *Synechococcus* growth not only when nitrate or nitrite is the nitrogen source but also when ammonium is the nitrogen source. Since the activities of the two enzymes involved in fixation of ammonium, GS and GOGAT, were higher in the *ntcB* mutant than in the wild-type strain (Table 2), *ntcB* is apparently not required for activation of the genes for GS and GOGAT.

While GS and GOGAT activities were higher in the mutant than in the wild-type strain, NR and NiR activities were lower in the mutant than in the wild-type strain (Table 2). Since many members of the LysR family activate a divergently transcribed operon located upstream, the lower NR and NiR activity suggests that NtcB is involved in activation of the *nirA* operon. However, the *nirA* transcript level in the *ntcB* mutant was similar to that in the wild-type strain (Fig. 6, lane 6). Thus, the involvement of *ntcB* in activation of the *nirA* operon is unlikely and the role of *ntcB* in *Synechococcus* nitrogen metabolism remains unclear.

TABLE 2. Enzyme activities involved in nitrogen assimilation in the wild-type and mutant strains[a]

Mutation	Avg enzyme activity[b] (range)			
	NR[c]	NiR[c]	GS[d]	GOGAT[d]
None (wild type)	261 (252–270)	94 (88–102)	35 (33–36)	0.51 (0.36–0.66)
nirB	392 (362–421)	37 (25–51)	38 (37–41)	0.72 (0.66–0.78)
ntcB	170 (153–186)	73 (66–81)	51 (49–53)	0.94 (0.80–1.08)

[a] Ammonium-grown cells of wild-type *Synechococcus* sp. strain PCC7942 and mutant strains were transferred to nitrate-containing medium, and the enzyme activities were assayed after 12 h of growth with nitrate.
[b] The values shown are averages from two (GOGAT) or three (NR, NiR, and GS) separate experiments.
[c] Activities are expressed in micromoles per milligram of chlorophyll per hour.
[d] Activities are expressed in micromoles per milligram of protein per hour.

DISCUSSION

In this study, we found two nitrogen-regulated genes, *nirB* and *ntcB*, in the DNA region upstream of the *nirA* operon required for uptake and assimilation of nitrate (28, 36). The two genes are oriented away from *nirA*, with *nirB* being located proximal to *ntcB* (Fig. 1 and 2). Similar to activation of the *nirA* operon (23), activation of *nirB* and *ntcB* depends on NtcA (Fig. 5), a global nitrogen regulator that activates the genes related to nitrogen assimilation under conditions of nitrogen limitation (23, 37). Luque et al. have identified three NtcA-binding sites with the palindromic consensus sequence $GTAN_8TAC$ (*nir*I, *nir*II, and *nir*III) in the region upstream of *nirA* (23). The present findings show that the NtcA-binding motifs are located symmetrically in the 286-bp region between the *nirA* and *nirB* coding regions (Fig. 2); the one proximal to *nirA* (*nir*I) is centered at −72.5 with respect to the coding region of *nirA*, and the one proximal to *nirB* (*nir*III) is centered at −74.5 with respect to the coding region of *nirB*, while the third (*nir*II) is centered at −141.5 and −145.5 with respect to the *nirA* ORF and the *nirB* ORF, respectively. Luque et al. (23) showed that NtcA-regulated promoters, including that of the *nirA* operon, have an NtcA-binding site and a −10 element with a TAN_3T consensus located 23 bases downstream of the NtcA-binding motif. According to this rule, NtcA-binding motif *nir*III and the sequence TTAGGT (nucleotides 232 to 237 in Fig. 2), which is located 23 bases downstream from *nir*III and 10 bases upstream of the transcription start site, are identified as the promoter elements of *nirB*, although the −10 motif does not exactly match the proposed consensus sequence. We suppose that genes *nirB* and *ntcB* are cotranscribed as a polycistronic mRNA because (i) the termination codon of *nirB* is located seven bases downstream of the start codon of *ntcB* and (ii) there is no NtcA-binding motif in the region immediately upstream of *ntcB*.

Defined mutants constructed by interrupting *nirB* or *ntcB* with a kanamycin resistance marker showed impaired growth in nitrate- or nitrite-containing medium (Table 1). When grown on ammonium, cultures of the *ntcB* mutant still showed a yellow-green color typical of nitrogen-limited cells but the *nirB* mutant grew normally (Table 1), indicating that *ntcB* is expressed in the *nirB* mutant. The gene cartridge used for interruption of *nirB* was C.K1 (9), which carries a kanamycin resistance gene with no transcription termination signal (4). We showed previously that insertion of C.K1 into *nrtD* in the *nirA* operon, in the same orientation as the gene does not inhibit expression of the *narB* gene located downstream (28). The absence of a negative polar effect on *ntcB* in the *nirB* mutant is therefore assumed to be due to the absence of a transcription termination signal on C.K1 and does not contradict the idea that *nirB* and *ntcB* form an operon.

Insertional inactivation of the *nirB* gene resulted in a decrease in in vitro NiR activity measured with methylviologen as the electron donor but not those of NR, GS, and GOGAT (Table 2). The *nirB* mutant excreted nitrite into the medium (Fig. 7), showing that the in vivo NiR activity is not high enough to reduce all of the nitrite produced by reduction of nitrate. By contrast, the *ntcB* mutant showed no specific decline in NiR activity nor excretion of nitrite into the medium (Table 2 and Fig. 7). These findings suggest that the *nirB* gene is required for expression of maximum NiR activity. It is clearly not essential for the biosynthesis and assembly of NiR, since appreciable NiR activity exists in the *nirB* insertion mutant (Table 2). The possible function of *nirB* includes (i) activation of the genes involved in biosynthesis and assembly of the cofactors of NiR, i.e., siroheme and the iron-sulfur cluster; (ii) facilitation of the assembly of functional NiR; and (iii) stabilization of functional NiR. Since the deduced NirB protein sequence has no similarities to known proteins, its biochemical function cannot be deduced from its primary structure. Biochemical studies are needed for elucidation of the function of NirB.

The deduced NtcB protein sequence has a structure typical of a LysR type of transcriptional activator with a helix-turn-helix DNA-binding motif near its N terminus (Fig. 3). Since activation of *ntcB* is dependent on *ntcA* (Fig. 5B), NtcB is supposed to activate another gene(s) in response to nitrogen limitation. However, existence of a low but significant level of the *ntcB* transcript in ammonium-grown cells (Fig. 4 and 5) and the yellow-green color of the ammonium-grown cultures of the *ntcB* mutant (Table 1) indicate that NtcB has a role under nitrogen-replete conditions as well. Since ammonium is fixed by GS as the amide nitrogen of glutamine and subsequently used for biosynthesis of various organic nitrogenous compounds, a mechanism seems to be required for regulating the distribution of the fixed nitrogen to different biosynthetic pathways. Operation of such a mechanism would be important for balanced utilization of the fixed nitrogen, especially under conditions of nitrogen limitation, in which various metabolic pathways compete for limited amounts of fixed nitrogen. The presumed role of *ntcB* in gene regulation and its activation under nitrogen-limited conditions suggest that the gene takes part in the regulation of partitioning of fixed nitrogen by controlling the activities of a certain biosynthetic pathway(s). Identification of the gene(s) regulated by NtcB is needed for elucidation of its role in the nitrogen metabolism of *Synechococcus* sp. strain PCC7942.

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