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Identification and Nitrogen Regulation of the Cyanase Gene from the Cyanobacteria *Synechocystis* sp. Strain PCC 6803 and *Synechococcus* sp. Strain PCC 7942

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An open reading frame (slr0899) on the genome of *Synechocystis* sp. strain PCC 6803 encodes a polypeptide of 149 amino acid residues, the sequence of which is 40% identical to that of cyanase from *Escherichia coli*. Introduction into a cyanase-deficient *E. coli* strain of a plasmid-borne slr0899 resulted in expression of low but significant activity of cyanase. Targeted interruption of a homolog of slr0899 from *Synechococcus* sp. strain PCC 7942, encoding a protein 77% identical to that encoded by slr0899, resulted in loss of cellular cyanase activity. These results indicated that slr0899 and its homolog in the strain PCC 7942 represent the cyanobacterial cyanase gene (designated *cynS*). While *cynS* of strain PCC 6803 is tightly clustered with the four putative molybdenum cofactor biosynthesis genes located downstream, *cynS* of strain PCC 7942 was found to be tightly clustered with the two genes located upstream, which encode proteins similar to the subunits of the cyanobacterial nitrate-nitrite transporter. In both strains, *cynS* was transcribed as a part of a large transcription unit and the transcription was negatively regulated by ammonium. Cyanase activity was low in ammonium-grown cells and was induced 7- to 13-fold by inhibition of ammonium fixation or by transfer of the cells to ammonium-free media. These findings indicated that cyanase is an ammonium-repressible enzyme in cyanobacteria, the expression of which is regulated at the level of transcription. Similar to other ammonium-repressible genes in cyanobacteria, expression of *cynS* required NtcA, a global nitrogen regulator of cyanobacteria.

Cyanase (EC 4.3.99.1), which catalyzes the decomposition of cyanate (NCO^-) into CO_2 and NH_3, has been shown to be present in plants, some heterotrophic bacteria, and the cyanobacterium *Synechococcus* sp. strain PCC 6301 (6, 22). In living organisms, cyanate is formed by the decomposition of carbamoylphosphate (CP) or urea (2, 14). Cyanate reacts with nucleophilic groups of proteins and hence is a toxic compound. With cyanase, however, *Escherichia coli* cells can utilize cyanate as the sole source of nitrogen (11). Cyanate has been also shown to reversibly inhibit CP synthetase of *E. coli* (4). The proposed biological functions of cyanase in *E. coli* therefore include detoxification of endogenously formed cyanate, utilization of cyanate as the nitrogen source (11), and regulation of enzyme activities through modulation of the intracellular cyanate level (12). For the cyanobacterium *Synechococcus* sp. strain PCC 6301, Miller and Espie found a high activity of cyanase and estimated the rate of cyanate decomposition per cell to be 20 times that in *E. coli* (22). The high cyanase activity implies the importance of cyanate metabolism in cyanobacterial cells, but the physiological role of the enzyme remains to be clarified.

In the investigation of the mechanism of ammonium-promoted activation and repression of the carbon and nitrogen assimilation genes, respectively, in *Synechococcus* sp. strain PCC 7942, we found that exogenously added cyanate strongly activates carbon assimilation genes while repressing nitrogen assimilation genes (30). In cyanobacterial cells, cyanate is supposed to arise from spontaneous dissociation of CP (17), which in turn is synthesized from glutamine, CO_2, and ATP (22). An intracellular CP pool was detected in ammonium-grown cells but not in N_2-grown cells of the diazotrophic cyanobacterium *Anabaena cylindrica* (17), which indicates that the endogenous level of CP, and hence of cyanate, would change according to the changes in the availability of ammonium in cyanobacteria. We therefore proposed that endogenous cyanate is the metabolic regulator of the ammonium-promoted gene regulation (30). However, the existence of a high, reportedly constitutive activity of cyanase in strain PCC 6301 (22), a close relative of strain PCC 7942, raises the question whether endogenously generated cyanate can affect gene expression in vivo. In the present study, we found that cyanase is an ammonium-repressible enzyme in *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942. The open reading frame (ORF) slr0899 of *Synechococcus* sp. strain PCC 6803 (16) and its homolog of *Synechocystis* sp. strain PCC 7942, encoding cyanase-like sequences, were identified as the cyanobacterial cyanase gene (designated *cynS*) and shown to be repressed by ammonium. The possible role of cyanase in the regulatory circuit of ammonium-promoted gene regulation is discussed.

MATERIALS AND METHODS

Strains and growth conditions. Cells of *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942 were grown photoautotrophically at 30°C under CO_2-sufficient conditions as described previously (30). The basal medium used was a nitrogen-free medium obtained by a modification of BG11 medium (27) described previously (30). 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. All media were buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 8.2). Transcription of the *cynS* gene and expression of cyanase activity were induced by treatment of ammonium-grown cyanobacterial cells with L-methionine-DL-sulfoximine (MSX), an inhibitor of ammonium fixation by glutamine synthetase,

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or by transfer of the ammonium-grown cells to ammonium-free media. MSX was added to cyanobacterial cultures in the mid-logarithmic phase of growth to a final concentration of 0.1 mM. For transfer of the cells to ammonium-free media, the ammonium-grown cells were collected by centrifugation at 5,000 × *g* for 5 min at 25°C, washed twice with the basal medium, and inoculated into the basal medium supplemented with KNO_3 or unsupplemented.

E. coli JM105 and DH5α, used as hosts for plasmid constructions, and a cyanase-less *E. coli* strain, CSH26 (23), used as the host for a genomic library of *Synechococcus* sp. strain PCC7942 constructed in pBluescriptII KS$^-$ and for expression of the cyanobacterial *cynS* gene, were grown on Luria-Bertani medium supplemented with ampicillin (50 μg/ml) and glucose (0.1%) when appropriate.

Expression of cyanobacterial *cynS* in *E. coli*. A 0.8-kbp fragment of *Synechocystis* sp. strain PCC 6803 DNA, carrying the entire slr0899 ORF with 280 bases and 56 bases, respectively, of its 5′- and 3′-flanking sequences, was excised from an M13mp18 clone carrying the ORFs slr0898 and slr0899 (16) and cloned between the *Pst*I and *Kpn*I sites of pBluescriptII SK$^+$ in the same orientation as that of *lacZ* on the vector. The resulting plasmid (pCY2), in which the slr0899 ORF was placed 250 bases downstream from a truncated LacZ ORF, was transformed into *E. coli* CSH26.

Cyanase assay. Cyanase activity in extracts of *E. coli* cells was assayed essentially as described by Guilloton et al. (13), except that the growth and assay temperature was 26°C. Cyanase activity in intact cyanobacterial cells was measured at 30°C as described by Miller and Espie (22) by determination of the ammonium formed by cyanase action on cyanate, which was excreted from the cells into medium in the presence of MSX. Since inhibition of ammonium fixation with MSX results in induction of the cyanase gene (see Results), chloramphenicol was added to the cell suspensions at 250 μg/ml to prevent de novo synthesis of cyanase during the assay.

Cloning and nucleotide sequence determination of the *cynS* region of *Synechococcus* sp. strain PCC 7942 DNA. For amplification of a *cynS* fragment of *Synechococcus* sp. strain PCC 7942, the PCR was carried out on approximately 200 ng of chromosomal DNA, with degenerate oligonucleotides synthesized according to the amino acid sequences conserved in the CynS proteins from *E. coli* and *Synechocystis* sp. strain PCC 6803, i.e., 5′-TA(C/T)CG(A/C/G/T)TT(C/T)TA(C/T)GA(A/G)AT-3′ and 5′-TA(C/T)AG(A/G)TT(C/T)TA(C/T)GA(A/G)AT-3′ for YRFYE(IM) (sense primers) and 5′-TC(A/C/G/T)CC(A/G)AA(C/T)TT(C/T)TC(A/G)TG-3′ for HEKFGD (antisense primer). The PCR fragment was used to isolate clones from a library of genomic DNA of *Synechococcus* sp. strain PCC 7942 that was constructed by ligating an *Eco*RI-*Hin*dIII digest of the genomic DNA in pBluescriptII KS$^-$. The plasmid purified from selected clones was found to contain a 0.47-kbp fragment of *Synechococcus* sp. strain PCC 7942 DNA carrying an ORF that was strongly similar to *cynS* (slr0899) of *Synechocystis* sp. strain PCC 6803. By use of the cloned DNA fragment, a mutant (CYN1) of strain PCC 7942, carrying a pBluescriptII KS$^+$ vector inserted into the putative *cynS* coding region, was constructed (see below). For cloning of the upstream and downstream regions of *cynS*, the chromosomal DNA from CYN1 was digested with *Pst*I and *Eco*47III, respectively, and the resulting fragments were circularized by ligation and transformed into *E. coli* DH5α. The plasmid thus rescued from the *Pst*I digest of the mutant genome contained a 2.1-kbp fragment of *Synechococcus* DNA, which carried 1.8 kb of the upstream region of the putative *cynS* gene as well as a copy of the gene that was truncated at the 3′ end. The plasmid rescued from the *Eco*47III digest of the mutant genome carried 7 kb of the downstream region of *cynS*. Nucleotide sequences of the cloned DNA fragments were determined by the dideoxy chain termination method. Searches through databases for sequence similarities of genes were performed with the BLAST program (3).

Insertional inactivation of *cynS* in *Synechococcus* sp. strain PCC 7942. A *cynS* insertional mutant (CYN1) of strain PCC 7942 was constructed as follows. A 0.2-kbp *Fok*I fragment of the putative *cynS* gene, corresponding to nucleotides 100 to 317 of the coding region, was cloned into the *Eco*RV site of pBluescriptII KS$^+$ after blunting of the termini. The orientation of the cloned *cynS* fragment was opposite to that of *lac* promoter on the vector. The kanamycin resistance gene cartridge C.K1 (9), carrying *nptII* (7), was subsequently ligated into the *Bam*HI site in the polylinker of the plasmid, so that the kanamycin resistance gene was in the same orientation as the *cynS* fragment. The resulting plasmid (pCY1) was used to transform *Synechococcus* sp. strain PCC 7942 to kanamycin resistance through single homologous recombination between the *cynS* fragment on the plasmid and the genomic copy of the gene (Fig. 1). 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 (34), genomic DNA was isolated from the selected clones and analyzed by Southern hybridization to confirm the presence and position of the pBluescript vector.

Isolation and analysis of DNA and RNA. Chromosomal DNA was extracted and purified from *Synechococcus* sp. strain PCC 7942 cells as described by Williams (34). Total RNA was extracted and purified from *Synechococcus* sp. strain PCC 7942 and *Synechocystis* strain PCC 6803 cells by the method of Aiba et al. (1). For Southern hybridization analysis of genomic DNA from the *cynS* insertional mutant of strain PCC 7942, DNA samples (5 μg per lane) were digested with *Nhe*I, fractionated on a 1.0% agarose gel, and transferred to a positively charged nylon membrane (Hybond N+; Amersham). For Northern hybridization analysis of the *cynS* transcripts, RNA samples (10 or 20 μg per lane) were denatured by treatment with formamide, fractionated by electrophoresis on 1.2% agarose gels that contained formaldehyde, and transferred to the positively charged nylon membranes. For dot hybridization analysis, 1.25- and 2.5-μg aliquots of each of the denatured RNA samples were spotted on the nylon membranes with a dot blot apparatus. The DNA and RNA blots were allowed to hybridize as described by Church and Gilbert (8) with the following gene-specific probes: a 0.47-kbp *Eco*RI-*Hin*dIII fragment of strain PCC 7942

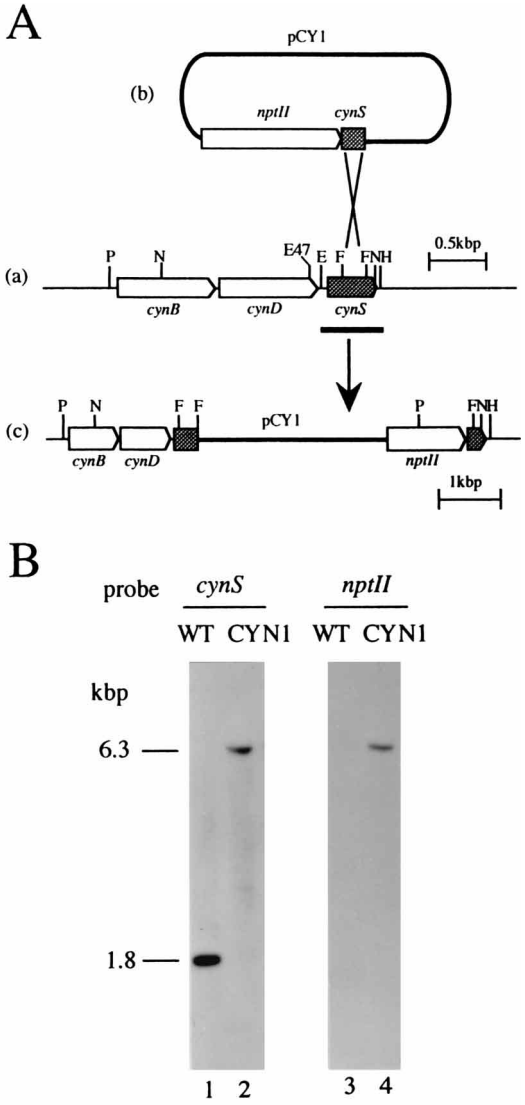

FIG. 1. (A) Schematic representation of the construction of a *cynS* insertional mutant (CYN1) of *Synechococcus* sp. strain PCC 7942. (a) Restriction map of genomic DNA around *cynS* of the wild-type *Synechococcus* sp. strain PCC 7942; (b) structure of the plasmid pCY1 carrying an internal fragment of *cynS*; (c) restriction map of the *cynS* region of the genome of mutant CYN1 resulting from integration of pCY1 into the genome through single homologous recombination between the chromosomal copy of *cynS* and the *cynS* fragment on pCY1. The shaded bars represent *cynS* and its fragments. The thick bar below the map in row a shows the *Eco*RI-*Hin*dIII fragment of genomic DNA which was used as a *cynS*-specific probe for Southern and Northern hybridization analyses. Restriction endonuclease site abbreviations: E, *Eco*RI; E47, *Eco*47III; F, *Fok*I; H, *Hin*dIII; N, *Nco*I; P, *Pst*I. The nucleotide sequence of a 2,500-bp fragment of genomic DNA, extending from the *Pst*I site located upstream of *cynB* to the base 268 downstream from the *cynS* termination codon, was determined in this study. (B) Southern hybridization analysis of genomic DNA from the wild-type strain (lanes 1 and 3) and the CYN1 mutant (lanes 2 and 4). DNA samples (5 μg per lane) were digested with *Nhe*I, fractionated on a 1.0% agarose gel, transferred to a positively charged nylon membrane (Hybond N+; Amersham), and hybridized with the ^{32}P-labeled gene-specific probes as indicated.

TABLE 1. Cyanase activity of *E. coli* JM105 and *E. coli* CSH26 carrying pCY2

Strain	Cyanase activity (nmol mg of protein^{-1} min^{-1})		
	Control	+IPTG	+KOCN
JM105 (*cynS*$^+$)	7	ND[a]	150
CSH26 with pBluescriptII SK$^+$	4	8	ND
CSH26 with pCY2 (containing slr0899)	47	44	ND

[a] ND, not determined.

DNA carrying the entire *cynS* gene; a 1.8-kbp *Pst*I-*Eco*RI fragment of strain PCC 7942 DNA carrying the two ORFs located upstream of *cynS*; a 0.46-kbp fragment of *Synechocystis* sp. strain PCC 6803 DNA carrying the entire *cynS* gene, excised with *Nco*I and *Kpn*I from the M13mp18 clone carrying the slr0898 and slr0899 ORFs (16); and the kanamycin resistance gene cartridge C.K1 (9) carrying *nptII*. The double-stranded DNA probes were labeled with ^{32}P as described by Feinberg and Vogelstein (10). The hybridization signals were detected by autoradiography on X-ray film or by a Bio-image analyzer (Fuji Photo Film). The radioactivity of the RNA dots was quantified with a Bio-image analyzer.

Analytical methods. Ammonium, chlorophyll, and protein were determined as described by Anderson and Little (5), Mackinney (19), and Lowry et al. (18), respectively.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB000100.

RESULTS

Expression of slr0899 in cyanaseless *E. coli*. A 0.8-kbp fragment of *Synechocystis* sp. strain PCC 6803 DNA, carrying 56 bases of the 3′ portion of the slr0898 ORF (putative nitrite reductase gene), the 224-base intergenic sequence between slr0898 and slr0899, the entire slr0899 ORF, and 56 bases of the 3′-flanking sequence of slr0899, was cloned between the *Pst*I and *Kpn*I sites of pBluescriptII SK$^+$ to construct plasmid pCY2. When the plasmid was transformed into the cyanaseless *E. coli* strain CSH26, low cyanase activity was detected in the cell extracts (Table 1). The activity was about 30% of that in the extracts from *E. coli* JM105 cells which had been treated with cyanate to induce expression of the endogenous cyanase gene but was much higher than the activity levels in the extracts from CSH26 transformed with pBluescriptII SK$^+$. The results suggested that slr0899 is the cyanase gene of *Synechocystis* sp. strain PCC 6803 (named *cynS* according to the designation for this gene in *E. coli*). Since cyanase activity was not increased by treatment with IPTG (isopropyl-β-D-thiogalactopyranoside) of the *E. coli* CSH26(pCY2) cells, expression of slr0899 was apparently not dependent on the *lac* promoter on the vector but was presumably driven by a promoter on the cloned cyanobacterial DNA. The absence of effects of IPTG on cyanase expression also suggested the presence of a transcription termination signal in the 224-base intergenic region between slr0898 and slr0899 that was present in pCY2.

Cloning of the *cynS* region of *Synechococcus* sp. strain PCC 7942 DNA. A 71-bp DNA fragment encoding an amino acid sequence strongly similar to a partial sequence of the CynS protein of *Synechocystis* sp. strain PCC 6803 was amplified from chromosomal DNA of *Synechococcus* sp. strain PCC 7942 by PCR and used for screening of a genomic library of the cyanobacterium constructed in pBluescriptII KS$^-$. A 471-bp *Eco*RI-*Hin*dIII fragment of strain PCC 7942 DNA thus cloned carried a 441-nucleotide ORF starting with an ATG codon, which encoded a CynS-like protein. After completion of the sequencing of the *Eco*RI-*Hin*dIII fragment, a nucleotide sequence of a 484-bp fragment of *Synechococcus* sp. strain PCC 7942 DNA carrying a putative cyanase gene (14a), to which our sequence was identical, appeared in the database. Cloning and nucleotide sequence analysis of the DNA region farther upstream of the *Eco*RI site showed that the *cynS*-like ORF is tightly clustered with the two ORFs located upstream (designated *cynB* and *cynD*) (Fig. 1). The *cynS*-like ORF was separated from the termination codon of *cynD* by only 10 bases and was preceded by a potential Shine-Dalgarno sequence (GGAG) overlapping the termination codon of *cynD*. The *cynD* ORF was separated from the termination codon of *cynB* by only 3 bases. These results suggested that *cynS* is cotranscribed with the upstream genes as an operon in *Synechococcus* sp. strain PCC 7942. There was no potential protein-coding regions within 200 bases downstream from the putative *cynS* gene.

To examine whether the *cynS*-like ORF represents the cyanase gene of *Synechococcus* sp. strain PCC 7942, a targeted mutant (CYN1) of the cyanobacterium, carrying two truncated copies of the putative *cynS* gene, was constructed by inserting a plasmid (pCY1) into the gene through single homologous recombination between the internal segment of the gene on pCY1 and the genomic copy of the gene (Fig. 1A). In Southern hybridization analysis of the *Nhe*I digests, the *cynS*-specific probe hybridized with a 1.8-kbp fragment of chromosomal DNA from the wild-type strain (Fig. 1B). The hybridizing fragment in the DNA from the mutant was 6.3 kbp in size, which was larger by 4.5 kbp, corresponding to the size of pCY1, than the hybridizing band in the wild-type DNA (Fig. 1B). The *nptII*-specific probe hybridized not with the DNA from the wild-type strain but with the 6.3-kbp fragment of the DNA from the mutant (Fig. 1B). These results indicate that pCY1 had been integrated into *cynS* on the chromosome of CYN1. While the nitrate-grown wild-type *Synechococcus* sp. strain PCC 7942 cells showed cyanase activity of 82 μmol per mg of chlorophyll per h (see below), the mutant showed no cyanase activity (<0.5 μmol per mg of chlorophyll per h) irrespective of the nitrogen source. The results indicated that the cloned gene is the sole cyanase gene of *Synechococcus* sp. strain PCC 7942. The CYN1 mutant grew normally in media containing nitrate or ammonium as the nitrogen source, showing that cyanase is not essential for growth of the cyanobacterium under the growth conditions tested.

Deduced amino acid sequences. The deduced CynS protein of *Synechococcus* sp. strain PCC 7942 consists of 146 amino acids and has a calculated molecular weight of 16,362. The CynS proteins from *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803 (16) are 77% identical to each other (Fig. 2). The cyanobacterial CynS proteins are only

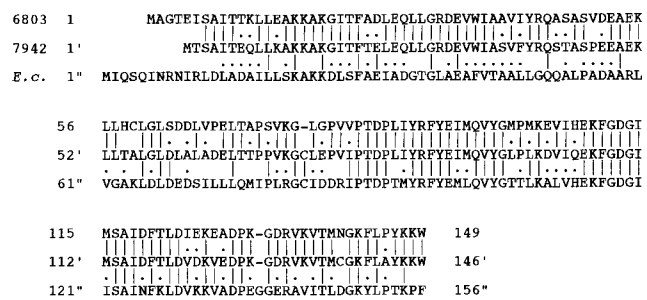

FIG. 2. Alignment of the deduced amino acid sequences of CynS from *Synechococcus* sp. strain PCC 7942 (7942), *Synechocystis* sp. strain PCC 6803 (6803), and *E. coli* (*E.c.*). The alignments were optimized by the FASTA program (26). Vertical lines indicate aligned and identical amino acid residues between the adjacent sequences. The dots indicate conservative replacements of amino acid residues.

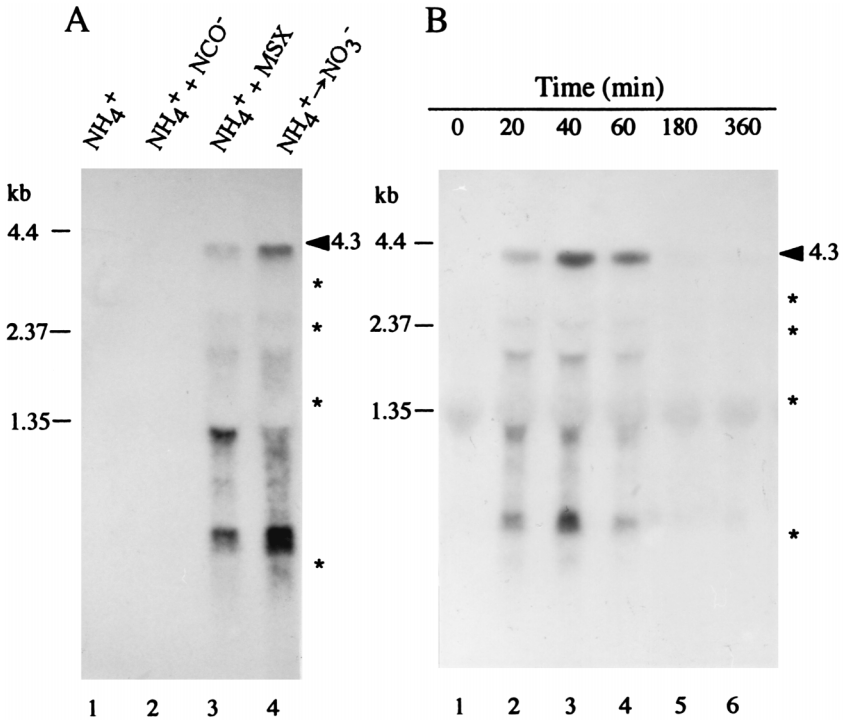

FIG. 3. Northern blot analysis of total RNA from wild-type *Synechococcus* sp. strain PCC 7942 with *cynS* as a probe. (A) Effects of different treatments of ammonium-grown cells on accumulation of the *cynS* transcript. Cells were grown with ammonium, the culture was separated into four portions, and total RNA was extracted from the cells before (lane 1) and 40 min after (lanes 2 to 4) the following treatments: addition of cyanate (lane 2), addition of MSX (lane 3), and transfer of the cells to nitrate-containing medium (lane 4). (B) Changes in the *cynS* transcript abundance after transfer of ammonium-grown cells to nitrate-containing medium. RNA samples were extracted before (lane 1) and after 20, 40, 60, 180, and 360 min of incubation in nitrate-containing medium (lanes 2 to 6, respectively). RNA (10 and 20 μg per lane in panels A and B, respectively) was denatured with formamide, separated on a 1.2% agarose–formaldehyde gel, transferred to a positively charged nylon membrane (Hybond N+; Amersham), and hybridized as described by Church and Gilbert (8) with the ^{32}P-labeled *cynS* from *Synechococcus* sp. strain PCC 7942. Asterisks indicate the positions of the rRNA bands as determined by staining of the blots with methylene blue (data not shown).

40% identical to the cyanase protein of *E. coli* (28), but the C-terminal portions of the cyanobacterial CynS proteins (amino acids 80 to 146 in *Synechococcus* sp. strain PCC 7942 and 83 to 149 in *Synechocystis* sp. strain PCC 6803) are 60% identical to that of *E. coli* (amino acids 89 to 156) since most of the conserved amino acid residues are located in the C-terminal portion of the proteins (Fig. 2). These findings suggested that cyanase contains more of the functionally essential amino acid residues in the C-terminal portion than in the N-terminal portion.

cynB and *cynD* encode proteins of 262 and 289 amino acids, respectively. The protein encoded by *cynB* is a hydrophobic protein similar to the integral membrane component of the ABC-type nitrate-nitrite transporter, NrtB, of the cyanobacteria *Synechococcus* sp. strain PCC 7942 (25), *Synechocystis* sp. strain PCC 6803 (gi | 1652570) (15), and *Phormidium laminosum* (21). It is also similar to CmpB, the integral membrane component of another cyanobacterial ABC transporter closely related to the nitrate-nitrite transporter, from strain PCC 7942 (gi | 11019379) (24) and strain PCC 6803 (gi | 1001317) (16). The extent of identity is 41 to 42% with the NrtB proteins and 39 to 40% with the CmpB proteins (alignment not shown). The protein encoded by *cynD* has ATP-binding motifs and is strongly similar to the ATP-binding subunits NrtD and CmpD of the two closely-related cyanobacterial ABC transporters mentioned above; it is 58 and 52% identical to the NrtD proteins of strain PCC 7942 (25) and strain PCC 6803 (gi | 1652568) (15), respectively, and 52 and 53% identical to the CmpD proteins of strain PCC 7942 (gi | 1019381) (24) and strain 6803 (gi | 1001320) (16), respectively (alignment not shown). The tight clustering of *cynB* and *cynD* and their deduced amino acid sequences suggest that the two ORFs encode subunits of an ABC transporter.

Transcription of *cynS* and its regulation. Northern hybridization analysis of total RNA from *Synechococcus* sp. strain PCC 7942 (Fig. 3A) showed that there was no detectable amount of the *cynS* transcript in ammonium-grown cells (lane 1). Unlike in heterotrophic bacteria, the addition of cyanate did not induce transcription of *cynS* (lane 2). Similar to the case of the *nirA* and *nirB* operons of strain PCC 7942, the addition of MSX to the ammonium-grown cells (lane 3) or transfer of the cells to nitrate-containing medium (lane 4) induced transcript accumulation. Induction of *cynS* transcription by MSX treatment in the absence of nitrate (lane 3) indicated that *cynS* is an ammonium-repressible gene which is activated simply by derepression. Similar to the case of the *nirA* and *nirB* operons, the level of *cynS* transcripts was highest at around 30 min after the transfer of ammonium-grown cells to nitrate-containing medium and then decreased (Fig. 3B), showing negative feedback by the ammonium generated internally by nitrate reduction. The *cynS* transcript was barely detectable after 180 min of incubation in nitrate-containing medium (Fig. 3B, lane 5).

The *cynS*-specific probe hybridized with an RNA 4.3 kb in size and also yielded signals corresponding to fragments extending from 0.2 to 2.5 kb (Fig. 3). The broad 1.5-kb band observed in both ammonium-grown cells and nitrogen-limited cells in some of the hybridization profiles (e.g., Fig. 3B) was

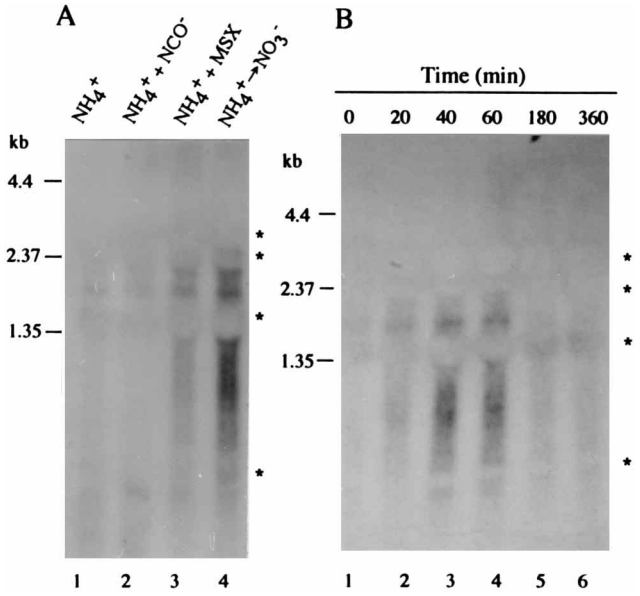

FIG. 4. Northern blot analysis of total RNA from *Synechocystis* sp. strain PCC 6803 with *cynS* as a probe. (A) Effects of different treatments of ammonium-grown cells on accumulation of the *cynS* transcript. Cells were grown with ammonium, the culture was separated into four portions, and total RNA was extracted from the cells before (lane 1) and 60 min after (lanes 2 to 4) the following treatments: addition of cyanate (lane 2), addition of MSX (lane 3), and transfer of the cells to nitrate-containing medium (lane 4). (B) Changes in the *cynS* transcript abundance after transfer of ammonium-grown cells to nitrate-containing medium. RNA samples were extracted before (lane 1) and after 20, 40, 60, 180, and 360 min of incubation in nitrate-containing medium (lanes 2 to 6, respectively). The amount of RNA used for the analysis was 10 μg per lane in panels A and B. Other experimental conditions were the same as those described in the legend to Fig. 3, except that *cynS* from *Synechocystis* sp. strain PCC 6803 was used as the probe. Asterisks indicate the positions of the rRNA bands.

presumably due to nonspecific binding of the probe to the 1.5-kb 16S rRNA band. The smaller-molecular-size signals other than the 1.5- and 4.3-kb bands were detected only in RNA samples from nitrogen-limited cells and ascribed to specific binding of the *cynS* probe to mRNA. Although the smaller-molecular-size signals included some diffuse bands, these were located just in front of, or at the back of, the huge rRNA bands in the gel, suggesting that they do not represent transcripts with distinct sizes but are artifacts arising from exclusion of mRNA by rRNAs. In the RNA samples purified successively from the same batch of culture (Fig. 3B), the intensity of the smaller-molecular-size signals correlated with that of the 4.3-kb signal, suggesting that the former have arisen from decomposition of the 4.3-kb transcript. A probe specific to *cynB* and *cynD* hybridized with the 4.3-kb RNA species as well as with the smaller-molecular-size bands, verifying the presence of an operon including *cynB*, *cynD*, and *cynS* (data not shown).

The *cynS* gene of *Synechocystis* sp. strain PCC 6803 is tightly clustered with the four putative molybdenum cofactor biosynthesis genes located downstream (16), suggesting the presence of an operon 4.3 kb in length. In the Northern hybridization analysis of RNA from strain PCC 6803, however, the probe specific to *cynS* of strain PCC 6803 did not yield a signal corresponding to 4.3 kb but yielded smeared hybridization signals extending from 0.25 to 2.4 kb, with the exclusion of radioactivity in the regions of the rRNA bands (Fig. 4, asterisks). The results yet indicated the presence of a large transcription unit including *cynS*, since the size of the hybridizing signal exceeded that of the *cynS* coding region. The absence of a large discrete hybridizing band indicated that the turnover rate of *cynS* transcript is much higher in *Synechocystis* sp. strain PCC 6803 than in *Synechococcus* sp. strain PCC 7942.

As observed in *Synechococcus* sp. strain PCC 7942, the *cynS* transcript of *Synechocystis* sp. strain PCC 6803 was hardly detectable in ammonium-grown cells (Fig. 4A, lane 1) and accumulated upon treatment of the ammonium-grown cells with MSX (lane 3) or upon transfer of the cells to nitrate-containing medium (lane 4). Also, the transcript accumulated transiently after the transfer of ammonium-grown cells to nitrate-containing medium and was barely detectable after adaptation of the cells to nitrate-containing medium (Fig. 4B). These results indicated that the *cynS* genes from *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942 are regulated in a similar manner with respect to the environmental conditions, although they are clustered and cotranscribed with distinct sets of genes.

Dependence of *cynS* transcription on *ntcA* in strain PCC 7942. Expression of ammonium-repressible genes of cyanobacteria generally requires NtcA, a Crp-type transcriptional activator protein (32). To determine whether transcription of *cynS* depends on NtcA, the levels of the *cynS* transcript in the wild-type strain and in an *ntcA* deletion mutant of strain PCC 7942 (29) were compared under the conditions for *cynS* induction (Fig. 5). Since the *ntcA* mutant cannot utilize nitrate as the nitrogen source (33), ammonium-grown cells of the wild-type and mutant strains were transferred to nitrogen-free medium to avoid possible influence of nitrate assimilation on *cynS* transcription in the wild-type cells. Under the inducing conditions, activation of *cynS* transcription occurred in the wild-type strain but not in the *ntcA* mutant (Fig. 5), showing that *cynS* transcription is dependent on NtcA in *Synechococcus* sp. strain PCC 7942.

Expression of cyanase activity in cyanobacterial cells. Table 2 compares the effects of various nutritional conditions on the in vivo activity of cyanase in *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803. In both strains, cyanase activity was low in ammonium-grown cells and high in nitrate-grown cells. Although the amount of *cynS* transcript in ammo-

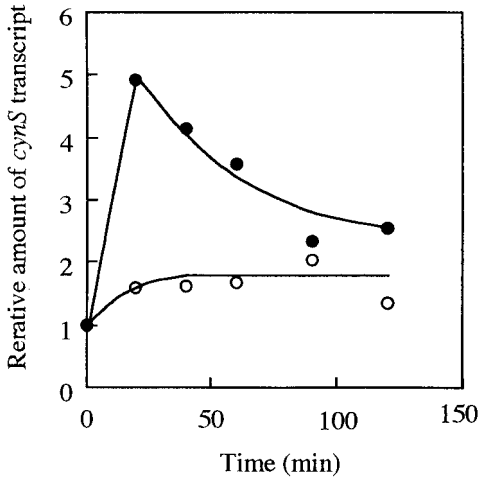

FIG. 5. Changes in the *cynS* transcript levels after transfer of ammonium-grown cells of the wild-type strain (●) and an *ntcA* deletion mutant (○) of *Synechococcus* sp. strain PCC 7942 to a nitrogen-free medium. The amounts of the *cynS* transcript were quantitated by dot hybridization analysis with 2.5 μg of RNA per dot and are shown relative to the maximum level in the wild-type cells. The *cynS*-specific probe used and the hybridization conditions were the same as those described in the legend to Fig. 3.

TABLE 2. Cyanase activity in the cells of *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803 grown under different nutritional conditions

Strain	Nutritional conditions	Cyanase activity[a]
Synechococcus sp. strain PCC 7942	Ammonium grown	14.3 ± 5.0 (n = 5)
	Ammonium grown, after MSX treatment[b]	96.7 ± 49.3 (n = 3)
	Ammonium grown, after transfer to nitrate-containing medium[c]	127.5 ± 65.9 (n = 3)
	Nitrate grown	82.2 ± 13.2 (n = 4)
Synechocystis sp. strain PCC 6803	Ammonium grown	4.2 ± 4.1 (n = 4)
	Ammonium grown, after MSX treatment[b]	54.1 ± 13.3 (n = 4)
	Ammonium grown, after transfer to nitrate-containing medium[c]	49.2 ± 26.7 (n = 4)
	Nitrate grown	54.4 ± 7.3 (n = 4)

[a] Activities are expressed in micromoles per milligram of chlorophyll per hour. The values shown are the averages ± standard deviations from separate experiments on independent cultures.
[b] MSX was added to the ammonium-grown cultures, and cyanase activity was assayed after 3 h of incubation under the growth conditions.
[c] Ammonium-grown cells were transferred to nitrate-containing medium, and cyanase activity was assayed after 3 h of growth.

nium-grown cells was below the limit of detection (Fig. 3), the low but significant cyanase activity in ammonium-grown cells of wild-type PCC 7942 cells (Table 2) was ascribed to the product of the *cynS* gene because ammonium-grown cells of the *cynS* mutant exhibited practically no cyanase activity (<0.5 μmol per mg of chlorophyll per h; see above). As expected from the transcriptional regulation of *cynS*, cyanase activity was induced 7- to 13-fold upon treatment of the ammonium-grown cells with MSX or upon transfer of the cells to nitrate-containing medium.

DISCUSSION

In *E. coli* and other bacteria, the expression of cyanase is induced by its substrate, cyanate (31). For the cyanobacterium *Synechococcus* sp. strain PCC 6301, a close relative of strain PCC 7942, Miller and Espie detected high cyanase activity in the cells grown in the absence of exogenous cyanate and assumed that cyanase is a constitutively synthesized enzyme (22). In accordance with their observations, expression of the cyanase gene, *cynS*, was not dependent on cyanate or induced by cyanate in the cyanobacteria *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803 (Fig. 3 and 4). The present results, however, revealed that *cynS* is not constitutively expressed but is negatively regulated by ammonium in the two strains of cyanobacteria; the levels of *cynS* transcription and cellular cyanase activity were low in ammonium-grown cells and were increased by inhibition of ammonium fixation or by transfer of the cells to ammonium-free media (Fig. 3 and 4; Table 2). In cyanobacteria, activation of the ammonium-repressible genes identified to date requires NtcA, a Crp-type global nitrogen regulator protein (32). The absence of stimulation of *cynS* transcription in an *ntcA*-deficient mutant of *Synechococcus* sp. strain PCC 7942 (Fig. 5) clearly demonstrates that *cynS* is an NtcA-dependent gene.

Since *Synechococcus* cells have been shown to assimilate the ammonium resulting from cyanase action on cyanate (22), the physiological role of cyanase in cyanobacteria probably includes utilization of exogenous cyanate as a nitrogen source. Cyanate is supposed to arise also from spontaneous dissociation of endogenous CP in cyanobacterial cells (17). CP is synthesized from Gln, CO_2, and ATP (20), and its concentration has been estimated to be 0.29 mM in ammonium-grown, nitrogen-replete cells and null in N_2-grown, nitrogen-limited cells of the diazotrophic cyanobacterium *A. cylindrica* (17). The rate of cyanate formation is therefore supposed to be high in ammonium-grown cells and low in nitrogen-limited cells. Since cyanase activity is low in ammonium-grown cells and high in nitrogen-limited cells (Table 2), detoxification of the endogenously formed cyanate is unlikely to be a major physiological role of cyanase in cyanobacteria.

The nitrogen regulation of cyanase expression seems to be consistent with our previous proposal that endogenous cyanate acts as a signal of nitrogen repletion, which activates and represses carbon and nitrogen assimilation genes, respectively (30); the low cyanase activity in ammonium-grown cells would allow accumulation of intracellular cyanate, while the high cyanase activity in nitrogen-limited cells keeps intracellular cyanate concentration null. It is reasonable to assume that the cyanate concentration in nitrogen-limited cells is close to zero. However, it is necessary to measure intracellular cyanate in ammonium-grown cells to evaluate our hypothesis, since the intracellular cyanate concentration in ammonium-grown cells is determined by the balance of the rates of cyanate formation and decomposition and is hardly predictable in the absence of knowledge of kinetic properties of cyanobacterial cyanase.

Unlike in *Synechocystis* sp. strain PCC 6803, in which *cynS* is tightly clustered with the four putative molybdenum cofactor biosynthesis genes located downstream (16), *cynS* of *Synechococcus* sp. strain PCC 7942 is tightly clustered with the two genes located upstream (Fig. 1), forming a part of a large transcription unit of 4.3 kb (Fig. 3). The strong similarities of the proteins encoded by the two genes to the subunits of the ABC-type nitrate-nitrite transporter, NrtB and NrtD (15, 25), and to the corresponding subunits of another closely related ABC transporter, CmpB and CmpD (16, 24), indicate that they also encode subunits of an ABC transporter which is likely to be involved in cyanate metabolism. Since cyanase requires bicarbonate as the second substrate for cyanate decomposition (22), the possible function of the two genes involves transport of bicarbonate as well as that of cyanate. Mutants of the *cynB* and *cynD* genes are being constructed to identify the substrate of the transporter.

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ADDENDUM IN PROOF

The nucleotide sequence of *cynA*, located immediately upstream of *cynB*, recently appeared in the databases (F. Jalali and G. S. Espie, GenBank accession no. AF001333, 1997).

Although *cynA* is apparently the first gene of an operon, *cynABDS*, the reported 5′-flanking sequence of *cynA* does not contain the consensus sequence of the NtcA-dependent promoters ($GTAN_8TACN_{22}TAN_3T$). We therefore sequenced farther upstream of the *cynA* translation start site and found a consensus sequence for an NtcA-dependent promoter (−334 to −293 with respect to the translation start site) (Y. Harano et al., DDBJ accession no. AB005890, 1997), consistent with our observation that *cyn* operon transcription is dependent on NtcA.

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