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 two 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 CO₂ and NH₃, 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 cyanohydrin, however, *Escherichia coli* cells can utilize cyanate as the sole source of nitrogen (11). Cyanate has been 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 modulations 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, CO₂, and ATP (22). An intracellular CP pool was detected in ammonium-grown cells but not in N₂-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 *Synechocystis* 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 N₂-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 (NH₄)₂SO₄ and 15 mM KNO₃, respectively, to the basal medium. All media were buffered with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH (pH 8.2).

The cyanS gene and expression of cyanase activity were induced by treatment of ammonium-grown cyanobacterial cells with L-methionine-cysteine-malonate (MSX), an inhibitor of ammonium fixation by glutamine synthetase.
or by transfer of the ammonium-growing 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-growing 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 KNO₃ 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 slr0898 region (200 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 into the pBluescriptII KS⁺ vector in the same orientation as that of lacZ on the vector. The resulting plasmid (pCY2), in which the srl0899 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 Guillot 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 amount of hydrated cyanate produced from cyanate, which was extracted 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 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′-TCATG(A/G)TCC(A/G)TCC(A/G)TCAATCATG(AG)TCATG(A/G)ATCATG(AG)TA-3′ and 5′-TCATG(A/G)TCCAAG(A/G)TCATG(A/G)TCC(A/G)TCAATG(A/G)TCC(A/G)TAATCATG(AG)TA-3′ for HEKFGD (antisense primer) and 5′-TCATG(A/G)TCC(A/G)TCC(A/G)TCAATG(A/G)TCC(A/G)TAATCATG(AG)TA-3′ for YRFYE(IM) (sense primers) and 5′-TCATG(A/G)TCCAAG(A/G)TCATG(A/G)TCC(A/G)TCAATG(A/G)TCC(A/G)TAATCATG(AG)TA-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 EcoRI-HindIII digest of the genomic DNA in pBluescript II 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 (srl0899) 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 PstI and Eco47III, respectively, and the remaining fragments were circularized by ligation and transformed into E. coli DH5α. The plasmid thus rescued from the PstI 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 Eco47III 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 FokI fragment of the putative cynS gene, corresponding to nucleotides 100 to 317 of the coding region, was cloned into the EcoRV site of pBluescriptI KS⁺ after bluntling of the termini. The orientation of the cloned cynS fragment was opposite to that of lacZ promoter on the vector. The kanamycin resistance gene cartridge C.K1 (9), carrying nptII (7), was subsequently ligated into the BamHI 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 (NH₄)₂SO₄. After three serial streak purifications to segregate homoygous mutants (34), genomic DNA was isolated from the selected clones and analyzed by Southern hybridization to confirm the presence and segregation 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 sp. strain PCC 7942 DNA samples (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 Ndel, 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 EcoRI-HindIII fragment of strain PCC 7942.
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 the *nptII* ORF. The double-stranded DNA probes were labeled with 32P 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 AB001000.

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 nitrile 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-*Hind*III 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-*Hind*III 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cyanase activity (nmol mg of protein−1 min−1)</th>
<th>Control</th>
<th>+IPTG</th>
<th>+KOCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM105 (cynS−)</td>
<td>7</td>
<td>ND a</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>CSH26 with pBluescriptII SK +</td>
<td>4</td>
<td>8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CSH26 with pCY2 (containing slr0899)</td>
<td>47</td>
<td>44</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

a ND, not determined.

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.
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

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**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). 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).
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 (10), 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 transcript 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 ammon-
Cyanate in the cyanobacteria *Synechococcus* anase gene, *PCC 7942*, Miller and Espie detected high cyanase activity in the *cynS* *Synechococcus* containing medium. grown cells with MSX or upon transfer of the cells to nitrate-

Ammonium-grown, after MSX treatment

Ammonium-grown, after transfer to nitrate-containing medium

Nitrate grown

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 are 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.

**ACKNOWLEDGMENTS**

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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 cytA is apparently the first gene of an operon, 

\textit{cytA\textit{ABDS}}, the reported 5’-flanking sequence of \textit{cytA} does not contain the consensus sequence of the NtcA-dependent promoters (GTAN\textsubscript{T}ACN\textsubscript{T}AN\textsubscript{T}). We therefore sequenced farther upstream of the \textit{cytA} 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 \textit{cyt} operon transcription is dependent on NtcA.

REFERENCES


