Involvement of a CbbR Homolog in Low CO₂-Induced Activation of the Bicarbonate Transporter Operon in Cyanobacteria

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Received 28 August 2000/Accepted 12 December 2000

The *cmpABCD* operon of *Synechococcus* sp. strain PCC 7942, encoding a high-affinity bicarbonate transporter, is transcribed only under CO₂-limited conditions. In *Synechocystis* sp. strain PCC 6803, the *slr0040*, *slr0041*, *slr0043*, and *slr0044* genes, forming an operon with a putative porin gene (*slr0042*), were identified as the *cmpA*, *cmpB*, *cmpC*, and *cmpD* genes, respectively, on the basis of their strong similarities to the corresponding *Synechococcus cmp* genes and their induction under low CO₂ conditions. Immediately upstream of and transcribed divergently from the *Synechocystis cmp* operon is a gene (*slI0030*) encoding a homolog of CbbR, a LysR family transcriptional regulator of the CO₂ fixation operons of chemoautotrophic and purple photosynthetic bacteria. Inactivation of *slI0030*, but not of another closely related *cbbR* homolog (*slI1594*), abolished low CO₂ induction of *cmp* operon expression. Gel retardation assays showed specific binding of the SlI0030 protein to the *slI0030-cmpA* intergenic region, suggesting that the protein activates transcription of the *cmp* operon by interacting with its regulatory region. A *cbbR* homolog similar to *slI0030* and *slI1594* was cloned from *Synechococcus* sp. strain PCC 7942 and shown to be involved in the low CO₂-induced activation of the *cmp* operon. We hence designated the *Synechocystis slI0030* gene and the *Synechococcus cbbR* homolog *cmpR*. In the mutants of the *cbbR* homologs, upregulation of ribulose-1,5-bisphosphate carboxylase/oxygenase operon expression by CO₂ limitation was either unaffected (strain PCC 6803) or enhanced (strain PCC 7942), suggesting existence of other low CO₂-responsive transcriptional regulator(s) in cyanobacteria.

Cyanobacteria fix CO₂ efficiently despite the low affinity and selectivity of their ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) for CO₂, because they possess a CO₂-concentrating mechanism (CCM) to elevate the CO₂ concentration around the active site of Rubisco (11, 21). The CCM involves the abilities to actively transport HCO₃[−] into the cell, to convert CO₂ to HCO₃[−] intracellularly, and to effectively convert HCO₃[−] into CO₂ in carboxysomes, the polyhedral inclusion bodies to which Rubisco is localized. It is supposed that the conversion of CO₂ to HCO₃[−] in the cytoplasm not only helps to maintain high intracellular HCO₃[−] concentrations but also allows diffusion of CO₂ from external medium into the cytoplasm (10). Biosynthesis of the components of CCM, including Rubisco, is supposed to be controlled by CO₂ availability (11, 21); however, detailed studies on the transcriptional regulation of the CCM-related genes are yet to be performed, and the underlying molecular mechanism is unknown.

Among the CCM-related genes, the *cmp* operon of *Synechococcus* sp. strain PCC 7942, encoding a high-affinity bicarbonate transporter (12, 18), is known to be a typical low CO₂-inducible transcription unit; the *cmp* operon mRNA and the CmpA protein, which is by far the most abundant protein

among the proteins encoded by the operon, are undetectable in cells grown under high CO₂ conditions (1 to 5%, vol/vol) and accumulate to a high level when the cells are transferred to CO₂-limited growth conditions (17, 18). To initiate studies on the regulation of the CCM-related genes, we identified a gene involved in the induction of *cmp* operon expression. Since genome sequence information is available for *Synechocystis* sp. strain PCC 6803 (9), we first identified the *cmp* operon and its regulator in this strain and then cloned and characterized the corresponding regulatory gene in *Synechococcus* sp. strain PCC 7942. It is shown that a homolog of *cbbR* (designated *cmpR*) plays an essential role in the low CO₂ induction of the *cmp* operon in the two strains of cyanobacteria. From the persistence of the low CO₂-responsive activation of Rubisco operon expression in the *cmpR* mutants, the existence of another mechanism for low CO₂-responsive gene activation in cyanobacteria is deduced.

MATERIALS AND METHODS

Strains and growth conditions. *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942, and mutants derived therefrom, were grown photoautotrophically under continuous illumination provided by fluorescent lamps (70 μmol of photons m^{−2} s^{−1}) at 30°C. The medium used was prepared by supplementing a nitrogen-free medium, obtained by modification of BG11 medium (27) as previously described (28), with 15 mM KNO₃. The medium was buffered with 20 mM HEPES-KOH (pH 8.0). When appropriate, kanamycin and spectinomycin were added to the medium at 15 μg ml^{−1}. The cultures were routinely maintained under high CO₂ conditions, i.e., aeration with 2% (vol/vol) CO₂ in air. For induction of *cmp* operon transcription, cells grown to the mid-logarithmic phase of growth were collected by centrifugation at 5,000 × g for 5 min at 25°C, washed twice with growth medium by resuspension and recentrifugation.

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A

B

FIG. 1. Gene organization in the *cmp* genomic region of *Synechocystis* sp. strain PCC 6803 and construction of insertional mutants of the *cbbR* homologs. (A) Map of the *cmp* region of the genome of *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803, showing the extent of identity of the deduced amino acid sequences of the corresponding *cmp* genes. (B) Structure of the *slr0030* genomic region of the MR1 and MR12 mutants and that of the *slr1594* genomic region of the MR2 and MR12 mutants of *Synechocystis* sp. strain PCC 6803. The gene organization in *Synechocystis* was obtained from Cyanobase (<http://www.kazusa.or.jp/cyano/cyano.html>). The *cmp* genes are indicated by stippled bars, and the *cbbR* homologs (*slr0030* and *slr1594*) are indicated by filled bars. Open bars represent the antibiotic resistance gene cassettes, with hatched bars showing the locations and orientations of the kanamycin resistance gene (*npt*) and the spectinomycin resistance gene (*aad*). Abbreviations for restriction endonuclease sites: B, *Bgl*II; E, *Eco*RV; N, *Nhe*I; D, *Dra*I.

gation, inoculated into fresh medium, and aerated with air containing ca. 0.005% (vol/vol) CO₂ under the same general conditions as before.

Insertional mutagenesis of *Synechocystis* *cbbR* homologs. A DNA fragment carrying the entire *slr0030* coding region (nucleotides +1 to +937 with respect to the translation start site) was amplified by PCR using the *Synechocystis* chromosomal DNA as the template and cloned into pT7Blue T-Vector. The forward primer used carried two additional nucleotides at the 5' end, which created a *Bsp*HI recognition sequence at the translational start site so that the cloned *slr0030* gene can be excised and used for construction of a translational fusion with maltose-binding protein (see below). After verification of the nucleotide sequence, a spectinomycin resistance cassette, which had been excised from plasmid pRL463 (3) by digestion with *Xba*I, was inserted at the *Nhe*I site in the cloned *slr0030* gene. Wild-type *Synechocystis* sp. strain PCC 6803 was transformed with the resulting plasmid to spectinomycin resistance through homologous recombination between the wild-type copy of *slr0030* on the genome and the interrupted copy of the gene, which resulted in replacement of the former with the latter to give rise to an *slr0030* insertional mutant (designated MR1) (Fig. 1B).

For construction of insertional mutants of *slr1594*, a DNA fragment carrying the entire *slr1594* coding region (nucleotides -1 to +962 with respect to the translation start site) with a base substitution from T to A at position +462 was generated by overlap extension PCR using oligonucleotide primers carrying mismatches with the wild-type sequence (8). The base substitution created a *Bgl*II recognition sequence in the *slr1594* coding region. After cloning of the DNA fragment into pT7Blue T-Vector and confirmation of the nucleotide sequence, a kanamycin resistance cassette excised from plasmid pUC4K (30) with *Bam*HI was inserted into the engineered *Bgl*II site to interrupt the cloned *slr1594* gene. The resulting plasmid was used to transform the wild-type *Synechocystis* strain and the MR1 mutant to kanamycin resistance through homologous re-

combination to obtain an *slr1594* insertional mutant (MR2) and an *slr0030* *slr1594* double mutant (MR12), respectively (Fig. 1B).

Identification, nucleotide sequence analysis, and insertional mutagenesis of a *Synechococcus* *cbbR* homolog. A 0.6-kbp fragment of a *cbbR* homolog was amplified from chromosomal DNA of *Synechococcus* sp. strain PCC 7942 by PCR, using degenerate oligonucleotides synthesized according to the amino acid sequences conserved in the two *Synechocystis* CbbR homologs encoded by *slr0030* and *slr1594*: 5'-TT(CT)AC(CGT)A(AG)(AG)GC(AGCT)GC(AGCT)GA(AG)GA-3' and 5'-TT(CT)AC(CGT)CG(AGCT)GC(AGCT)GC(AGCT)GA(AG)GA-3' for FT(RK)AAEE (forward primer), and 5'-GC(CT)TG(CT)TT(AG)A T(ACGT)GC(CT)TC(AG)TT-3' for NEAIKQA (reverse primer). For amplification of the DNA regions contiguous to the 0.6-kbp internal segment of the *cbbR* homolog, 1-μg aliquots of *Synechococcus* chromosomal DNA were digested with *Bam*HI and *Nhe*I, respectively, the resulting fragments were circularized by self-ligation, and the circularized DNA fragments were used as the templates for inverse PCR. DNA fragments of 6 and 2.5 kbp were obtained from the *Bam*HI and *Nhe*I digests, respectively, and used for determination of the nucleotide sequences of the 5' and 3' portions of the *cbbR* homolog and its 5' and 3' flanking regions.

For insertional interruption of the *cbbR* homolog in *Synechococcus* sp. strain PCC 7942, a DNA fragment carrying nucleotides -260 to +739 with respect to the translation start site was amplified by PCR and cloned into pT7Blue T-Vector. After confirmation of the nucleotide sequence, the spectinomycin resistance cassette from pRL463 was inserted into the *Msc*I site to interrupt the coding region. The resulting plasmid was used to transform the wild-type *Synechococcus* strain to spectinomycin resistance through homologous recombination to yield an insertional mutant (MR4) of the *cbbR* homolog.

Preparation of the *Synechocystis* CmpR protein and gel shift assay. The PCR-amplified *slr0030* gene cloned into pT7Blue T-Vector (see above) was excised

from the plasmid with *Bsp*HI and *Bam*HI and, after blunting of the termini, cloned into the *Sal*I site in the polylinker of the expression vector pMAL-c2 (22). The resulting plasmid, designated pMR1, carried a chimeric gene encoding a translational fusion of the maltose-binding protein with CmpR. Cells of *Escherichia coli* NM522 transformants carrying pMR1 were grown in Luria-Bertani medium. Expression of the chimeric gene was induced by 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG), and the recombinant protein was purified on amylose resin (22). The purified fusion protein was cleaved with factor Xa and used for gel retardation assays.

The DNA fragment used as the probe, carrying the entire intergenic region between *cmpA* and *cmpR* (nucleotides -252 to -1 with respect to the *cmpA* start codon), was labeled at both termini with ³²P, using the Klenow fragment of DNA polymerase I and [α -³²P]dCTP. Gel retardation assays were performed essentially as described by Buratowski and Chodosh (2). Gels containing 4% polyacrylamide were used for separation of the free probe and the protein-DNA complexes. Gels were dried, and the signals were detected by autoradiography.

Isolation and analysis of DNA and RNA. Chromosomal DNA was extracted and purified from *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942 cells as described by Williams (31). Manipulations and analyses of DNA were performed according to standard protocols (24). For Southern hybridization analysis of the genomic DNA digests, the following ³²P-labeled double-stranded DNA probes were used: a 0.92-kbp entire coding region of *slr0030*; a 0.95-kbp entire coding region of *slr1594*; a 0.59-kbp *Hinc*II fragment of *Synechococcus* *cmpR*; a 2-kbp spectinomycin resistance cassette; and a 1.3-kbp kanamycin resistance cassette. Total RNA was extracted and purified from the cyanobacterial cells by the method of Aiba et al. (1). For Northern hybridization analysis, RNA samples (10 μ g per lane) were denatured by treatment with formaldehyde, fractionated by electrophoresis in 1.2% agarose gels that contained formaldehyde, transferred to positively charged nylon membranes (Hybond N+; Amersham), and hybridized with the following gene-specific probes: a 737-bp PCR-amplified *rbcL* fragment of strain PCC 6803, carrying bases +50 to +786 of the coding region; a 3.8-kbp PCR-amplified fragment of strain PCC 6803 DNA, carrying 1,147 bases of *cmpA*, the entire *cmpB* gene, and 1,704 bp of *slr0042* (bases +213 to +4079 with respect to the translation start site of *cmpA*); a 0.71-kbp *Sal*I-*Sph*I fragment of *cmpC* of strain PCC 7942; a 17-mer oligonucleotide complementary to bases +1566 through +1582 of the *rbcL* coding region of strain PCC 7942; and a 288-bp PCR-amplified *ccmK* fragment of strain PCC 7942, carrying bases +11 to +298 of the coding region. The double-stranded DNA probes were labeled with ³²P as described by Feinberg and Vogelstein (4). The oligonucleotide probe was labeled with ³²P, using [γ -³²P]ATP and T4 polynucleotide kinase. The radioactivity of the probes that hybridized to mRNA was quantified with a Bio-Image analyzer (Fuji Photo Film).

Transformation of cyanobacteria. Transformation of *Synechocystis* and *Synechococcus* was performed as described by Williams (31). The transformants were allowed to grow on solid medium supplemented with kanamycin and/or spectinomycin at 15 μ g ml⁻¹ (see above). After three serial streak purifications to segregate homozygous mutants (31), genomic DNA was isolated from the selected clones and analyzed by Southern hybridization to confirm the presence and position of the antibiotic resistance gene.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession number AB047379.

RESULTS

Construction of *Synechocystis* mutants lacking the *cbhR* homologs. The *cmpA*, *cmpB*, *cmpC*, and *cmpD* genes of *Synechococcus* sp. strain PCC 7942, encoding the high-affinity bicarbonate transporter BCT1, form a low CO₂-inducible operon (18). These genes are similar, respectively, to the *nrtA*, *nrtB*, *nrtC*, and *nrtD* genes of the same cyanobacterium, which encode the nitrate/nitrite transporter (15, 16). Among the genes of *Synechocystis* sp. strain PCC 6803 (9), *slr0040*, *slr0041*, *slr0043*, and *slr0044* are the most similar to *cmpA*, *cmpB*, *cmpC*, and *cmpD*, respectively (Fig. 1A). They are more similar to the *cmp* genes than to the *nrt* genes of *Synechococcus*, although they were initially designated "*nrt*" genes in Cyanobase (<http://www.kazusa.or.jp/cyano/cyano.html>). On the other hand, another set of *Synechocystis* "*nrt*" genes in Cyanobase, *slr1450*,

slr1451, *slr1452*, and *slr1453*, having the second-best similarities to the *cmp* genes among the *Synechocystis* genes, are more similar to the *nrt* genes than to the *cmp* genes of *Synechococcus* and are in fact involved in nitrate/nitrite uptake (M. Kobayashi and T. Omata, unpublished results). Northern hybridization analysis showed that *slr0040*, *slr0041*, *slr0043*, and *slr0044* constitute a low CO₂-inducible operon (see below) with a putative porin gene, *slr0042* (6). On the basis of these observations, we have identified *slr0040*, *slr0041*, *slr0043*, and *slr0044* as the *cmpA*, *cmpB*, *cmpC*, and *cmpD* genes, respectively, of the *Synechocystis* strain. While there is no potential protein-coding region within 700 bases upstream from the *cmp* operon of *Synechococcus* (11), a gene (*slr0030*) coding for a protein 31 to 51% identical to bacterial CbbR (RbcR) proteins is located 253 bases upstream of the *Synechocystis* *cmp* operon, being oriented divergently from the *cmp* operon (Fig. 1A). CbbR is a LysR family protein involved in transcriptional activation of the Rubisco operon in chemoautotrophic and phototrophic bacteria (5, 26). Since many of the LysR family proteins activate the divergently transcribed operon located upstream (7, 25), *slr0030* was likely to be involved in activation of the *cmp* operon in *Synechocystis*.

To determine the role of *slr0030*, we constructed an insertional mutant of this gene. Since the *Synechocystis* genome contains two other *cbhR* homologs, *slr1594* and *slr0998*, encoding proteins 53 and 33% identical to the Slr0030 protein, respectively, insertional mutagenesis was attempted also for these two genes. In the case of *slr0030* and *slr1594*, the insertional inactivated genes segregated to give rise to homozygous mutants MR1 and MR2 (Fig. 1B), respectively, as determined by Southern hybridization analysis (data not shown). By inactivating *slr1594* in MR1, an *slr0030* *slr1594* double mutant MR12 was also constructed (Fig. 1B). Since segregation of the mutant genome, carrying the interrupted copy of the gene, was not attained for *slr0998* (data not shown), the mutants of *slr0030* and *slr1594* (MR1, MR2, and MR12) were further characterized.

Under photoautotrophic conditions, the three *cbhR* mutants grew slightly more slowly than the wild-type strain in the presence of both high (2%) and low (0.035%) CO₂ (data not shown). The final cell density in the mutant cultures was close to that in the cultures of the wild-type strain, indicating that *slr0030* and *slr1594* are not essential for growth of *Synechocystis* under the given conditions.

Activation of the *cmp* operon in the *Synechocystis* mutants. Figure 2 shows the effects of CO₂ conditions on transcription of the *cmp* operon and *rbc* operon in the wild-type and mutant *Synechocystis* strains. In the case of the wild-type strain, transfer of the cells to low CO₂ conditions induced accumulation of the *cmp* operon transcript (Fig. 2A, lanes 1 and 2). The hybridization profile showed a smeary signal ranging from 2 to 7 kb in size, indicating that the primary transcript is rapidly turned over. The low CO₂-induced accumulation of the *cmp* transcript was abolished in the MR1 and MR12 mutants lacking *slr0030* (lanes 3, 4, 7, and 8), but the MR2 mutant, which is defective solely in *slr1594*, showed normal low CO₂-induced accumulation of the *cmp* operon transcript (lanes 5 and 6). These results indicated that *slr0030* but not *slr1594* is essential for the low CO₂-responsive activation of the *cmp* operon in

FIG. 2. Northern blot analysis of total RNA from the wild-type strain (WT; lanes 1 and 2), the *slr0030* insertional mutant (MR1; lanes 3 and 4), the *slr1594* insertional mutant (MR2; lanes 5 and 6), and the *slr0030 slr1594* double mutant (R12; lanes 7 and 8) of *Synechocystis* sp. strain PCC 6803, showing the effects of CO₂ conditions on expression of the *cmp* and *rbc* operons. Cells were grown under high CO₂ conditions (2% CO₂ in air) and transferred to low CO₂ conditions (0.005% CO₂ in air); total RNA samples (10 µg per lane) were extracted from the cells before (lanes 1, 3, 5, and 7) and 90 min after (lanes 2, 4, 6, and 8) the transfer and analyzed, using probes specific to *cmpA-cmpB-slr0042* (A) and *rbcL* (B). Arrowheads indicate calculated sizes (in kilobases) of the full-length mRNA of the *cmp* operon (A) and the *rbc* operon (B).

Synechocystis. On the basis of these results, we named *slr0030* as *cmpR*.

Transfer of the wild-type *Synechocystis* cells to low CO₂ conditions also caused an increase in the abundance of the mRNA for the *rbcLXS* (*slr0009-slR0011-slR0012*) operon (Fig. 2B, lanes 1 and 2). The extent of the increase in the mRNA abundance was 60 to 70% in three separate experiments (data not shown). The low CO₂-induced increase in the *rbcLXS* mRNA abundance was observed also in the *slr0030* and *slr1594* mutants (lanes 3 to 8; 60 to 70% increase in MR1 and MR2 and 55 to 80% increase in MR12 in three separate experiments). Thus, neither gene was likely to be involved in regulation of *rbcLXS* expression.

Gel shift assay. To examine whether CmpR binds to the *cmpA* operon regulatory region, gel shift assays were performed with CmpR, using a DNA fragment carrying the *cmpR-cmpA* intergenic region as the probe. Since His₆-tagged CmpR was insoluble and could not be easily solubilized, CmpR was expressed in a soluble form as a MalE-CmpR fusion in *E. coli* (Fig. 3A, lanes 1 and 2), purified to near homogeneity (lanes 3

FIG. 3. Preparation of recombinant CmpR and the mobility shift assays. (A) Expression in *E. coli* and purification of the MalE-CmpR fusion (lanes 1 to 4) and cleavage of the MalE-CmpR fusion with factor Xa (lane 5). Proteins were separated on a sodium dodecyl sulfate–10% polyacrylamide gel and stained with Coomassie brilliant blue. Lane 1, total protein from the *E. coli* expression strain before IPTG treatment; lane 2, total protein from the expression strain after 2-h treatment with IPTG; lane 3, soluble fraction from the IPTG-induced expression strain; lane 4, the protein purified on amylose resin; and lane 5, the MalE-CmpR fusion cleaved with factor Xa. (B) Mobility shift assays showing retardation in a 4% polyacrylamide gel of the ³²P-labeled *cmpR-cmpA* intergenic segment by CmpR. Samples of the MalE-CmpR fusion (lane 1) and the MalE-CmpR fusion cleaved with factor Xa (lane 2 to 9) were added to the reaction mixtures to give the indicated final concentrations; 100-fold-excess amounts of nonlabeled *cmpR-cmpA* intergenic segment and a 0.7-kb segment of *rbcL* coding region were added to lanes 8 and 9, respectively, as competitors. C1 and C2, DNA-protein complexes; F, free probe.

and 4), cleaved from MalE with factor Xa (lane 5), and used for the experiments (Fig. 3B). While addition of the MalE-CmpR fusion up to a concentration of 1 µM did not affect the electrophoretic mobility of the DNA probe (Fig. 3B, lane 1), addition of the CmpR-MalE mixture to give CmpR and MalE concentrations higher than 10 nM yielded two retarded bands representing DNA-protein complexes (lanes 3, 4, 5, and 7). Addition of an excess amount of cold DNA probe prevented binding of the labeled probe to the protein (lane 8), but addition of a nonhomologous DNA fragment had no effect thereon (lane 9). No change in mobility of the DNA probe was observed when a MalE-LacZα mixture, obtained by cleavage of the MalE-LacZα fusion expressed from the vector (pMAL-c2), was used in place of the MalE-CmpR mixture for the experi-

FIG. 4. (A) Map of the genomic region of the *cbbR* homolog in *Synechococcus* sp. strain PCC 7942. The filled bar represents the *Synechococcus cbbR* homolog. The triangle above the map indicates the *MscI* site where a 2.0-kbp gene cassette carrying the spectinomycin resistance gene (*aad*) was inserted to construct the MR4 mutant. Abbreviations for restriction endonuclease sites: H, *HincII*; M, *MscI*. (B) Southern blot analysis of DNA from the wild-type strain (WT; lanes 1 and 3) and the MR4 mutant (lanes 2 and 4) of *Synechococcus* sp. strain PCC 7942. DNA samples (5 µg per lane) were digested with *HincII*, fractionated on a 0.7% agarose gel, transferred to a positively charged nylon membrane (Hybond N+; Amersham), and hybridized with the ³²P-labeled gene-specific probes as indicated.

ments (data not shown). These results showed that the CmpR protein specifically binds to the *cmpR-cmpA* intergenic region. Thus, CmpR is likely to activate transcription of the target operon by interacting with its promoter region, as other LysR-type transcription activators do (25).

Identification and nucleotide sequence determination of a *cbbR* homolog of *Synechococcus* sp. strain PCC 7942. Although functionally related genes are usually clustered on the genome of *Synechococcus* sp. strain PCC 7942, no *cmpR*-like gene was found in the region upstream of its *cmp* operon (Fig. 1A). This raised a question as to whether a *cbbR* homolog is involved in regulation of the *cmp* operon in this strain of cyanobacterium. Using degenerate oligonucleotides synthesized according to the amino acid sequences conserved in the proteins encoded by *sll0030* and *sll1594* (see Materials and Methods), a 0.6-kbp fragment was amplified from chromosomal DNA of *Synechococcus* sp. strain PCC 7942 by PCR and shown to encode a

FIG. 5. Alignment of the deduced amino acid sequence of the *cbbR* homolog of *Synechococcus* sp. strain PCC 7942 with those of the proteins encoded by *sll0030* (*cmpR*) and *sll1594* of *Synechocystis* sp. strain PCC 6803. The alignments were optimized by the FASTA program (19). Vertical lines indicate aligned and identical amino acid residues between adjacent sequences; dots indicate conservative replacements of amino acid residues.

CbbR homolog, as confirmed by cloning and nucleotide sequence determination, verifying the existence of a *cbbR*-like gene in *Synechococcus* sp. strain PCC 7942. Using the set of primers, only one species of *cbbR* homolog was amplified. A 2.5-kbp *NheI* fragment and a 6-kbp *BamHI* fragment of *Synechococcus* DNA, carrying the regions contiguous to the PCR-amplified 0.6-kb segment of the *cbbR* homolog, were subsequently amplified by inverse PCR and used for nucleotide sequence determination of the entire *cbbR* homolog and its flanking regions. Figure 4A shows the map of the DNA region around the *Synechococcus cbbR* homolog thus obtained. A homolog of *slr2141*, a *Synechocystis* gene of unknown function, was found to be located 71 bases upstream from the *cbbR* homolog. The open reading frame located farther upstream, which overlaps the *slr2141* homolog by four bases, was partially sequenced from its 3' end and tentatively identified as a tryptophanyl-tRNA synthetase gene (*trpS*) of *Synechococcus* sp. strain PCC 7942, because the encoded amino acid sequence was 73% identical to the C-terminal portion of tryptophanyl-tRNA synthetase from *Synechocystis* sp. strain PCC 6803. There was no potential protein-coding region within 600 bases downstream from the *cbbR* homolog. These results suggested that the *cbbR* homolog is the last gene of an operon. The organization of genes around the *Synechococcus cbbR* homolog is thus totally different from those in *Synechocystis* (Fig. 1B). The open reading frame of the *cbbR* homolog starts with a GTG codon and encodes a protein of 323 amino acids (see entry AB047379 in DDBJ, EMBL, and GenBank nucleotide sequence databases) which is 54.2 and 59.4% identical to the proteins encoded by *sll0030* and *sll1594*, respectively (Fig. 5).

FIG. 6. Northern blot analysis of total RNA from the wild-type strain (WT; lanes 1, 2, 5, 6, 9, and 10) and the insertional mutant of the *cbbR* homolog (MR4, lanes 3, 4, 7, 8, 11, and 12) of *Synechococcus* sp. strain PCC 7942, showing the effects of CO₂ conditions on expression of the *cmp*, *rbc*, and *ccm* operons. *Synechococcus* cells were grown under high CO₂ conditions (2% CO₂ in air) and transferred to low CO₂ conditions (0.005% CO₂ in air), and total RNA was extracted from the cells before (lanes 1, 3, 5, 7, 9, and 11) and 30 min after (lanes 2, 4, 6, 8, 10, and 12) the transfer. RNA samples (10 µg per lane) were denatured, fractionated by electrophoresis, transferred to positively charged nylon membranes, and hybridized with probes specific to *cmpC* (lanes 1 to 4), *rbcL* (lanes 5 to 8), and *ccmK* (lanes 9 to 12). Arrowheads indicate calculated sizes (in kilobases) of the full-length mRNA of the *cmp* (A), *rbc* (B), and *ccm* (C) operons.

Functional analysis of the *cbbR* homolog of *Synechococcus*.

To determine whether the *Synechococcus cbbR* homolog is involved in regulation of the *cmp* operon, a mutant (MR4) was constructed by inserting a spectinomycin resistance cassette in the gene (Fig. 4). Similar to the case of *Synechocystis*, a homozygous mutant lacking the wild-type gene was obtained (Fig. 4B). The mutant grew as fast as the wild-type strain under both high (2%) and low (0.035%) CO₂ conditions (data not shown), indicating that the *cbbR* homolog of *Synechococcus* is not essential for growth of the cyanobacterium under the given conditions.

Figure 6 compares the effects of CO₂ conditions on expression of the three transcription units of *Synechococcus*, i.e., the *cmp* operon, the *rbc* operon, and the carboxysome-related gene cluster *ccmKLMNO*, in the wild-type strain and the MR4 mutant. In the case of the wild-type strain, transfer of the cells to low CO₂ conditions induced accumulation of the *cmp* operon transcript as previously shown (18) (lanes 1 and 2). The mRNA from the *rbcLS* operon was detected in high CO₂-grown cells, and its abundance was increased by transfer of the cells to low CO₂ conditions (lanes 5 and 6). While only low-molecular-size signals of <1.4 kb were detected with the *ccmK*-specific probe in the mRNA samples from high CO₂-grown cells (lane 9),

CO₂ limitation caused accumulation of abundance of larger signals (lane 10). The maximum size of the hybridization signal, 3.8 kb (lane 10), corresponded to the entire size of the *ccmKLMNO* gene cluster, verifying that the five genes are transcribed as an operon at least under the CO₂-limited conditions. Unlike the *cmpR* mutants (MR1 and MR12) of *Synechocystis*, the *Synechococcus* MR4 mutant accumulated the *cmp* operon transcript in response to CO₂ limitation (compare lanes 3 and 4), but the level of the transcript was much lower than that in the wild-type strain (lane 2). On the other hand, the abundance of the transcripts from the *rbc* operon and the *ccm* operon was greater in the mutant than in the wild-type strain after induction (compare lanes 6 and 10 with lanes 8 and 12, respectively). The extent of low CO₂-responsive increase in levels of the *cmp*, *rbc*, and *ccm* transcripts was variable in different experiments, but that of the *rbc* and *ccm* transcripts was always larger in the MR4 mutant than in the wild-type strain (Fig. 7B and C), and the maximum level of *cmp* operon transcript in the mutant was insignificant compared to that in the wild-type strain (Fig. 7A), being 10 to 25% of the wild-type level. It should also be noted that the mutant often accumulated higher levels of *rbc* and *ccm* operon transcripts than the wild-type strain even under the high CO₂ conditions (Fig. 7B

FIG. 7. Changes in relative abundance of the *cmpC*, *rbcL*, and *ccmK* transcripts after transfer of the wild-type strain and the *cmpR* insertional mutant (MR4) of *Synechococcus* sp. strain PCC 7942 from high (2%) to low (0.005%) CO₂ conditions. Results of the dot hybridization analysis with 2.5 µg of RNA per dot were quantified and plotted. A representative of three sets of essentially the same results, obtained with three independent sets of cultures, is shown.

and C). The specific reduction in the level of *cmp* operon expression in the MR4 mutant indicated that the *cbbR* homolog of *Synechococcus* is involved in activation of the *cmp* operon. We therefore identified this gene as the *cmpR* gene of *Synechococcus* sp. strain PCC 7942.

DISCUSSION

The CbbR protein of chemoautotrophic and photosynthetic bacteria is required for activation of the operons encoding the enzymes of the Calvin-Benson-Bassham (CBB) cycle (5, 26). As is usually the case with the LysR family transcription regulator proteins (7, 25), the genes encoding CbbR are located immediately upstream of their target operons (5). While a single *cbbR* gene is present in most of these bacteria (5), the genome sequencing project of the cyanobacterium *Synechocys-*

tis sp. strain PCC 6803 revealed the presence of three genes coding for CbbR-like proteins, two of which (*sll0030* and *sll1594*) are more similar to the *cbbR* genes of chemoautotrophic and phototrophic bacteria (31 to 54% identity at the amino acid sequence level) than the third one (*sll0998*) is (25 to 33% identity) (9). None of the cyanobacterial *cbbR* homologs is, however, clustered with other *cbb* genes, and their functions remain to be determined. The present results indicate that the *sll0030* gene product activates the divergently transcribed operon located upstream, encoding the bicarbonate transporter, presumably by binding to its regulatory region (Fig. 3). Identification of a *Synechococcus cbbR* homolog involved in activation of the *cmp* operon (Fig. 4 to 7) suggests that cyanobacteria commonly utilize a CbbR homolog for the low CO₂ induction of the bicarbonate transporter operon. Since the transporter is not a component of the CBB cycle, we have named the *sll0030* gene and the *cbbR* homolog of *Synechococcus* sp. strain PCC 7942 as *cmpR*.

Rubisco activity (20) and the cellular carboxysome content (14, 29) have been shown to increase during adaptation of *Synechococcus* strains to low CO₂ conditions, and elevated expression of the *rbcLS* and *ccmNO* genes following transition from high to low CO₂ conditions has been reported in strain PCC 7942 (23). The present results confirm the latter observation and further show that the *ccmKLMNO* genes form an operon (Fig. 6). The upregulation of the *rbcLS* and *ccmKLMNO* operons was not abolished by inactivation of *cmpR* but rather enhanced by the mutation (Fig. 6 and 7). Low CO₂-induced increase in the abundance of *rbcLXS* mRNA was observed also in *Synechocystis* sp. strain PCC 6803 and was found to be unaffected by the mutation of *cmpR* and *sll1594* (Fig. 2). These findings suggest the presence of a *cmpR*-independent (and, in *Synechocystis*, *sll1594*-independent as well) mechanism for low CO₂-induced activation of the *rbc* and *ccm* operons. The transcriptional regulator(s) involved in this process is not known, but the third *cbbR* homolog of *Synechocystis* sp. strain PCC 6803 (*sll0998*), which could not be completely deleted from the cell, is a good candidate for the regulator, because its close homolog has been found not only in the cyanobacteria *Synechococcus* sp. strain PCC 7942 (78% identity at the amino acid sequence level [T. Omata and S. Gohta, unpublished results, GenBank accession no. AB053349]), *Anabaena* sp. strain PCC 7120 (82% identity [Cyanobase]), *Nostoc punctiforme* (83% identity [http://www.jgi.doe.gov/JGI_microbial/html/nostoc_homepage.html]), and *Synechococcus* sp. strain WH8102 (64% identity [http://www.jgi.doe.gov/JGI_microbial/html/synechococcus.html]) but also in cyanelle and chloroplast DNA from all known eukaryotes having *rbcS* in an operon with *rbcL* (51 to 72% identity) (summarized in reference 13; see GenBank entry NC001675 for the cyanelle sequence). Much more work is required to obtain a comprehensive view of the mechanism of low CO₂-induced gene regulation in cyanobacteria.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research (09640768) and Grants-in-Aid for Scientific Research in Priority Areas (09274101 and 09274103) to T.O. from the Ministry of Education, Science, Sports and Culture, Japan.

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