Transcriptional Regulation of the CO$_2$-Concentrating Mechanism in a Euryhaline, Coastal Marine Cyanobacterium, *Synechococcus* sp. Strain PCC 7002: Role of NdhR/CcmR[▽]

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Cyanobacterial photosynthesis occurs in radically diverse habitats and utilizes various forms of a CO$_2$-concentrating mechanism (CCM) featuring multiple inorganic carbon (C$_i$) transporters. Cyanobacteria from dynamic environments can transform CCM activity depending on C$_i$ availability, and yet the molecular basis for this regulation is unclear, especially in coastal strains. LysR family transcription factors resembling the Calvin cycle regulator CbbR from proteobacteria have been implicated in the expression of C$_i$ transporter genes in freshwater cyanobacteria. Our survey of related factors revealed a group of divergent CbbR-like sequences confined to freshwater and coastal or offshore cyanobacteria. Inactivation of the single gene (termed *ccmR*) from this variable cluster in the euryhaline (coastal) strain *Synechococcus* sp. strain PCC 7002 led to constitutive expression of a high-affinity CCM. Derepression of HCO$_3^-$ transporter gene transcription, including that of BicA, a recently discovered HCO$_3^-$ transporter (G. D. Price et al., Proc. Natl. Acad. Sci. USA 101:18228-18233, 2004), was observed. A unique CcmR-regulated operon containing *bicA* plus 9 open reading frames encoding likely Na$^+$/H$^+$ antiporters from the CPA1 and Mnh families was defined that is essential for maximal HCO$_3^-$-dependent oxygen evolution. The promoter region required for C$_i$-regulated transcription of this operon was defined. We propose that CcmR (and its associated regulon) represents a specialization for species inhabiting environments subject to fluctuating C$_i$ concentrations.

Cyanobacteria inhabit a vast range of aquatic or damp environments including arctic, freshwater, hypersaline, deep-sea (euphotic), coastal, and soda lake environments plus lichen symbioses. Cyanobacteria are subject to a significant restriction on their rates of photosynthesis owing to the relatively low availability of CO$_2$ in aquatic environments and have evolved several adaptations (collectively known as a "CO$_2$-concentrating mechanism" [CCM]) to facilitate efficient capture and fixation of CO$_2$ (reviewed in references 3, 19, and 23). Generically, the cyanobacterial CCM features multiple inorganic carbon transport systems for the active uptake of both CO$_2$ and HCO$_3^-$. Other key features include the cytosolic accumulation of inorganic carbon (C$_i$) as HCO$_3^-$ at significant disequilibrium from CO$_2$ (cytosolic carbonic anhydrase [CA] is absent) and the localization of the CO$_2$-fixing enzyme Rubisco inside microcompartments known as the carboxysomes. Inside the carboxysome, CO$_2$ is generated from HCO$_3^-$ in proximity to Rubisco by the activity of a specifically localized CA. However, there is considerable niche-related genetic diversity among the cyanobacteria with regard to the systems and components that comprise their CCM (4). An emerging area in this field is the study of the regulation of these systems. The extent to which the few known regulatory mechanisms are conserved or modified across ecotypes represents a new area of research in cyanobacterial comparative genomics.

The activity of the cyanobacterial CCM in the studied freshwater organisms *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803 and the euryhaline (coastal/estuarine) strain *Synechococcus* sp. strain PCC 7002 is strongly C$_i$ responsive and is fully engaged under C$_i$-limiting conditions. The apparent photosynthetic affinity for C$_i$, as measured by $K_{0.5}$(C$_i$), ranges between a low-affinity, constitutive state (approximately 200 μM) under C$_i$-replete conditions and a high-affinity, induced state (10 to 15 μM; reviewed in references 3, 8, and 19) under C$_i$ limitation. In freshwater strains, the transition between these states is characterized by dynamic and large changes in the expression and activity of inducible HCO$_3^-$ and CO$_2$ transport systems (11, 14–16, 20, 23, 28, 29). The signal response pathways that control CCM activity in response to C$_i$ availability are incompletely understood, particularly in euryhaline and marine strains of cyanobacteria. In the freshwater species *Synechococcus* sp. strain PCC 7942 a strong correlation between CCM activity and transient fluctuations in the C$_i$ pool has been established, as well as a dependence on oxygen for full expression of CCM activity (30). The downstream events in the C$_i$ signaling pathway of freshwater species has been better characterized, and LysR-type transcription factors are known to be involved in the regulation of high-affinity C$_i$ transporter gene expression as both activators and repressors (7, 15, 28).

In the euryhaline cyanobacterium *Synechococcus* sp. strain PCC 7002, two Na$^+$-dependent inducible HCO$_3^-$ transport activities have been characterized (20): a high-affinity, low-flux

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TABLE 1. Sequences of the gene-specific primers used in RT-PCR assays of the *ccmR* genomic region of *Synechococcus* strain PCC 7002[a]

Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
1350F	CTTTTGTCCAGAGAACCATACTC	5806F	GACAGTCACACCGCCTATTAC
2002R	GCAAATTACTCAGGATGTAGAGATC	6253R	ACTTGTTCCGCAATGTACAAC
1521F	GCACAAGTTCAGGAGTAAACCC	7172F	GTCGATGTACAACGTCACCTC
2876R	AATGCTGGAAATATCGAGGTC	7602R	AGTGAGAATACGCGCTAACTG
3215R	CAAAATTCCAAGTACCAGCAAG	7589F	CGCGTATTCTCACTTATCACG
4670R	ATACCAGCATCTTGGAGGTTG	8288R	CGTTTATGGCTCAAAACAGTG
1950F	GATCGGATGGCGGATAATC	8273F	GCCAAAGGAAAGTGCTTATAG
4228F	ACACAGTTATTTTTGCCGTTG	8726R	GAAGCGCAATTTTTATTGAAG

[a] Coordinates as in Fig. 4; GenBank accession no. U97516.

system, originally characterized in *Synechocystis* sp. strain PCC 6803 (23), which is encoded by the *sbtA* gene and a medium-affinity, high-flux transporter termed BicA, which is widely distributed across cyanobacterial species. A gene encoding a putative HCO_3^- porin (*porB*; the *Synechocystis* sp. strain PCC 6803 homologue [slr0042] is part of a known HCO_3^- transporter operon [15]) is strongly C_i responsive also in *Synechococcus* sp. strain PCC 7002 (20). The active transport of CO_2 in cyanobacterial species including *Synechococcus* sp. strain PCC 7002 is associated with specialized NDH-1 dehydrogenase complexes that are proposed to convert CO_2 to HCO_3^- within the cell. The *ndhF4*, *ndhD4*, and *chpX* (*cupB*) genes are required for a low-affinity constitutive CO_2 transport activity termed NDH-1$_4$, whereas an inducible, high-affinity CO_2 transporter, NDH-1$_3$, requires expression of the *ndhD3*, *ndhF3*, and *chpY* (*cupA*) genes (9, 10, 12, 13, 24). The role of LysR transcription factors in CCM regulation is currently unclear in this organism.

Euryhaline cyanobacterial strains such as *Synechococcus* sp. strain PCC 7002, which occur in coastal or estuarine environments, would certainly be subject to habitat fluctuations such as light, nutrients (especially C_i) and temperature owing to the injection of freshwater and nutrients from land, and tidal influences (4). Consequently, a regulatory system would be required to modulate CCM activity. In the present study, we have examined the genomic structure and transcriptional regulation of low-CO_2 induced C_i uptake systems in *Synechococcus* sp. strain PCC 7002, revealing some unique and compact gene organization. Signifying the importance of transcriptional controls on CCM activity in this system, we generated a mutant of a LysR family transcriptional regulator that exhibited complete derepression of CCM activity under C_i-replete conditions. Based on this discovery, it would be reasonable for this LysR factor to be termed CcmR, for CCM regulator, as suggested by Wang et al. (26).

MATERIALS AND METHODS

Cyanobacterial strains and culture conditions. *Synechococcus* sp. strain PCC 7002 cells (mutant and wild-type strains) were cultured as described by Price et al. (20) with a light intensity of approximately 85 μmol of photons $m^{-2} s^{-1}$. For the Δ*ccmR* and *ccmR* mutants, kanamycin was included at a final concentration of 150 μg ml^{-1}. To induce a medium- to high-affinity CCM rapidly, exponentially growing cells (optical density at 730 nm of 0.3 to 0.4), which had been bubbled with an air-CO_2 mixture containing 2% (vol/vol) CO_2, were harvested by centrifugation at 4,800 × *g* for 6 min and resuspended in an equivalent volume of modified BG-11 medium containing a final concentration of approximately 0.1 mM C_i before transfer to bubbling with air or CO_2-free air, respectively. The Δ*ccmR* deletion mutant was constructed sequentially. First, a pUC18-based construct consisting of the 0.90- and 0.93-kb regions immediately upstream and

downstream of *ccmR* was assembled. The primers for amplifying the upstream sequence were forward (5′-TTGAGCTCATCTAGGGCTTGGCGATC) and reverse (5′-TTGGATCCTCCTGAACTTGTGCTGTTATG) introducing the SacI and BamHI sites at the 5′ and 3′ ends, respectively. The primers for amplifying the downstream flanking sequence were forward (5′-TAGGATCCTTTCCCGTGCCTTTGGTAG) and reverse (5′-AAAAGCTTAATCAGCACCCAGGCTCCAG) introducing the BamHI and HindIII sites at the 5′ and 3′ ends, respectively, for assembly inside pUC18. A kanamycin resistance marker gene from the Tn*903* transposon (GenBank X06404) was cloned as a BamHI fragment into the resulting BamHI site in both transcriptional orientations relative to the *ccmR* flanking sequences. The cartridge was used to transform wild-type *Synechococcus* strain PCC 7002 cells as described by Sültemeyer et al. (26), and segregation was confirmed by PCR analysis. The construct for generating a *ccmR* insertional mutant was assembled in pGEMT. The primers for amplifying the *ccmR* open reading frame (ORF) were forward (5′-AAGGATCCAAGTTCAGGAGTAAACCCATGATC) and reverse (5′-AGCGGCCGCCAACAATTTTGAGCTTTTAGGG). A kanamycin resistance marker from the Tn*903* transposon was cloned into the XbaI site in the 5′ half of the ORF in the forward transcriptional orientation relative to the *ccmR* sequence. The cartridge was used for transformation of wild-type cells as described above.

The Δ*nha* mutant was made by assembling a construct in vector pGEMT (Invitrogen, Carlsbad, CA). A region from 860 bp upstream to 810 bp downstream of the SmaI and XbaI sites in *nhaS3* and *mnhD1*, respectively (see Fig. 5A), was amplified by PCR, and the section internal to the SmaI and XbaI sites (Fig. 5A) was replaced by cloning of a chloramphenicol acetyltransferase gene (conferring chloramphenicol resistance). The primers used to amplify the flanking sequences were as follows: forward, 5′-AATTCTATGCTAAGTCCCACAATCTTTTC, and reverse, 5′-AATTGCGGCTGGATAACTGTCC. Segregation of the Δ*nha* allele from wild-type alleles after three rounds of chloramphenicol selection was confirmed by PCR (results not shown).

RIF treatment of cyanobacterial cultures. High-C_i cells in mid-exponential growth phase were transferred to CO_2-free air for 30 min to induce high-affinity C_i transporter gene expression as previously described (20). At this point (designated time zero), two cultures were supplemented with 5 mM NaHCO₃ and swapped to bubbling with 1.7% CO_2 in the presence or absence of 200 μg of the transcriptional inhibitor rifampin (RIF) ml^{-1} for a further 2 h. The remaining two cultures were supplemented with 5 mM NaCl to balance sodium and bubbled with CO_2-free air with or without RIF as described above. All four cultures were sampled throughout this period.

Use of genome and protein databases. *Synechococcus* sp. strain PCC 7002 sequences were accessed from the complete genome database constructed by Donald Bryant and Tao Li (Pennsylvania State University) and Jindong Zhao (College of Life Sciences, Peking University) and are available at GenBank (for accession numbers, see Table 3). For other cyanobacterial sequences, gene object identifiers (GOIs) from the Integrated Microbial Genomes database (DOE, Joint Genome Institute; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) were used in preference to GenBank accession numbers if locus tags were unavailable. Information about the databases used to detect conserved protein domains is available at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml.

Reverse transcription-PCR (RT-PCR) assays. TAQ-Ti DNA polymerase kit components (Fisher-Biotech, West Perth, Australia) were used in 20-μl reactions containing 1.5 mM MgCl₂, 200 μM concentrations each of dATP, dTTP, dCTP and dGTP, 0.5 μM concentrations each of forward and reverse primer, 0.5 U of *Taq*, and cDNA template equivalent to 25 ng of total RNA. First-strand cDNA synthesis from normalized total RNA (derived from wild-type cells bubbled with air for 60 min and isolated according to the method of McGinn et al. [11]), was synthesized as described in Woodger et al. (29) using the set of reverse primers

TABLE 2. Sequences of the gene-specific primers used in RT-PCR assays of the *bicA* genomic region of *Synechococcus* strain PCC 7002[a]

Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
1279F	AGTTTTGCCATTGTAACTCG	8486R	GAGAAAAATTAAGCCTGGAGTC
1980R	TGACATGAAGCCAGAAATCAC	8333F	GATTAAAGATTGGCTCCATGTG
1462F	GATATTAAACTATGCTCCTGTTGC	8774R	ACAAAATTAGGACACCGACAAC
1561F	TGATCTGAAAAATGCGTAACC	8613F	GCGTTACTACTCCCGATTTATG
3198F	GGAAGAAGCCCTCAAGAATG	9081R	CACATACCCCAGTACCGTATTC
3700R	GAGACCCCGACTAGAACACC	8923F	CGTTCTGTGTTGTTTAAATTGC
4697F	TGATCTTGACCACTTTCTTGG	9325R	GAGGGTAATGGATAACATCGTC
5247R	ATTATCCCGTGACAACTTCATC	9510F	AAATGTGCACTGTATTTTGCAG
5061F	GGTGATCGCGTATTACATTCTC	9976R	GGGAATCGGTAAATTGCTTAAC
5462R	TCCAAGAGTTCTAGGGTAAAAGG	10140F	TGCCATTTATAAAAAGTGGAATG
6718F	TGGCTTAGGACAACTCTCAGTG	10539R	CAAACCGTATGCTGGTATGAC
7128R	AACGTAGAGGCTAATCAGATCG	10181F	TTTCGGTACTGGTCTTTTTCC
8014F	TATCTCGAAGCCTATCAACTGG	10692R	AGCACCGATTTTTCATTTTTC

[a] Coordinates are as in Fig. 5A; GenBank accession no. AF381039.

listed in Tables 1, 2, and 3. Thermocycling was performed in a TGradient thermal cycler (BIOMETRA, Goettingen, Germany) for 28 to 30 cycles consisting of denaturation for 15 s at 94°C, annealing at 54°C for 30 s, and extension at 72°C for 60 s. Cycling was preceded by a 5-min 94°C activation step. Primer pair sequences are listed in Tables 1, 2, and 3. For every reaction an RNA sample without reverse transcriptase was included to control for genomic DNA contamination, along with a positive control containing 5 to 50 ng of genomic DNA as a template. Products were separated by electrophoresis of 10 μl of each reaction on a 1.2% agarose gel containing 0.09 M Tris-borate–0.002 M EDTA (pH 8.0) with 0.5 mg of ethidium bromide ml^{-1} and visualized on a UV transilluminator.

Real-time quantitative RT-PCR assays. First-strand cDNA synthesis from normalized total RNA, isolated according to the method of McGinn et al. (11), was conducted as described in Woodger et al. (29). Quantitative real-time RT-PCR assays, using primer pairs specific to the *bicA* operon, *sbtA*, *ndhR*, and the *ndhF3 ndhD3 chpY orf133* operon and incorporating SYBR green I to monitor product formation, were performed as described by Woodger et al. (29). Primer sequences are listed in Table 3 or as described by Price et al. (20). The *rnpA* housekeeping gene was used in normalization as described by Price et al. (20). All reactions were carried out in quadruplicate, and errors were determined by standard methods.

Determination of TSPs. Transcription start points (TSPs) for *bicA* and *ndhF3* were determined by RNA ligase-mediated amplification of full-length 5′ cDNA ends (RLM-RACE) using the Generacer kit (L1500-1502; Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. In brief, total RNA was isolated from wild-type cells bubbled with air for 60 min (as described above). First-strand cDNA was generated from 3 μg of dephosphorylated RNA with full-length mRNA subsequently decapped and ligated to a specific RNA oligonucleotide. Nested PCR was performed, and specific products cloned and sequenced in the vector pCR4Blunt-TOPO. The following primers were used: *ndhF3* reverse, 5′-GCCCATTCAGCCAGACCATCAAA; *ndF3* reverse nested,

TABLE 3. Sequences of the gene-specific primers used in quantitative RT-PCR assays for *Synechococcus* strain PCC 7002[a]

Clone	GenBank accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
ndhF3	U97516	ACCGCGATGTATCTTTATCTC	ACAAAATAGCGGTCTAACCAG
ndhD3	U97516	CGTGTTCCAAGGTAGCTTTAG	AAAAGGATAATGACCGTCAAG
chpY	U97516	AAATTATTCCCAAATCCAAAG	GCATACAAAATACCGTAGTGG
ORF133	U97516	TGCCAAGTATTGTGGATATTG	TAAGTGAGAATACGCGCTAAC
ORF133+	U97516	CGCGTATTCTCACTTATCACG	TGAAACCGGAAAATTTGAAAC
sbtA	DQ632590	CAGCGATTGTTGTAGCTAGTC	AATTTCAACCCGATTAGTAGC
porB	DQ632588	TTACAAGCCTACAATTTTTCG	CCGTTAATTTATCTGTGATCG
bicA	AF381039	GTTCTTTTGTTTCAACTCAGTG	GCTTAAAGAGCTTGAGTTTTTC
nhaS3	AF381039	GATTTCGTTCCTTCCCTGTC	GAGACCCCGACTAGAACACC
mnhC	AF381039	ATCGCGTATTACATTCTCGTG	ATTATCCCGTGACAACTTCATC
mnhD1	AF381039	GGGATAATCCGACCTTAGAAAC	TCCAAGAGTTCTAGGGTAAAAGG
mnhD2	AF381039	AGAGCGGCTATTTTATTTTGAC	AACGTAGAGGCTAATCAGATCG
HCP 1	AF381039	GATTAAAGATTGGCTCCATGTG	ACAAAATTAGGACACCGACAAC
HCP 2	AF381039	GCGTTACTACTCCCGATTTATG	ACAAAATTAGGACACCGACAAC
HCP 3	AF381039	CGTTCTGTGTTGTTTAAATTGC	CACATACCCCAGTACCGTATTC
HCP 4	AF381039	CCTTGTTATTGCCCGTGAC	AACATCGTCCCCACTAACG
mnhB	AF381039	CTTTATTTGTGAAAATGCTCGTC	GGGAATCGGTAAATTGCTTAAC
mnhB+	AF381039	TGCCATTTATAAAAAGTGGAATG	CAAACCGTATGCTGGTATGAC
rbcS	AF015889	TGATGGATCAAGGCTATATTCC	AACAACACGGATGTAGCAGTC
rbcX	AF015889	AACAACACGGATGTAGCAGTC	ACTCACCAGGGTTAGGTTCAG
rbcL	AF015889	TACTTGGACCACTGTATGGAC	TCAAAACGTTGGTTACAGAAC
ccmK1	AF015889	CTATTGCAGTCGGAAGTGGTAG	GGAAGCTTGAACGTTCAGAAAC
ccmK2	AF015889	CGTGGTGATGTTTCTGAAGTC	ACTTCTTCGGTGTAGCGAATC
ccmL	AF015889	CAGCACCTACAAAGCAGAAAG	GTATCAATGATGCCAATGACC
ccmM	AF015889	TCGAATTTAATTGGTGATGTC	ATCCAGACAGAATAGTCATGG
ccmN	AF015889	AAATGCCTGCTTAGGTTATGG	TTGTGGCAGTCTGGTTAGTTG
ccmO	To be assigned	ACAACTGGCCTCCTATGAAAC	TTGGTTCTGCCTTATCTAGCC
ccaA	To be assigned	TATGCCTTTGGTTTACGATTG	ATCCAGCCATAGATTTTCAGC
p450	U97516	CTGACCCGATGGACATTTAC	AGCAGATAAATTGACCCCATC

[a] HCP, hypothetical conserved protein from the *bicA* operon (nomenclature as per Fig. 5).

TABLE 4. Sequences of the primers used to amplify *Synechococcus* sp. strain PCC 7002 DNA fragments from the upstream regions of the *ndhF3* [*ndhF3*(P550)] and *bicA* genes (722F/R and clones 2 to 12) for analysis as *luxAB* fusions in the integration vector pMBB

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
ndhF3(P550)	GAAAAATCTCCCTCTGTCTGTGTTGGATG	CACCATGACGATGACGCTGAAATAATTTG
bicA(P722F)	GCTATGGTCGATGGCGTTGAGTAAC	CACCTGTGGGTTCGGAAATGAG
bicA(P722R)	CACCGTCGATGGCGTTGAGTAAC	CTGTGGGTTCGGAAATGAGGG
Clone 2	GCTATGGTCGATGGCGTTGAGTAAC	CACCTCTGCATGGTTACGCATTTTTC
Clone 4	GCCCAGGGTGGCAATGGTTAGAC	CACCTCTGCATGGTTACGCATTTTTC
Clone 5	GCCCAGGGTGGCAATGGTTAGAC	CACCAGTGCAACAGGAGCATAGTTAATATC
Clone 7	CATAGGCGATCGCCACCTATGTTTTAG	CACCTCTGCATGGTTACGCATTTTTC
Clone 8	AGGGGGCTATGGTTAGATGTTAATCAATAC	CACCTCTGCATGGTTACGCATTTTTC
Clone 9	TATGCTCCTGTTGCACTGACAACTTAAC	CACCTCTGCATGGTTACGCATTTTTC
Clone 10	AATTCATCAATAAAGATAGTTTTCCCAATG	CACCTATCTATGAAAATTTTATTTTGTATTG
Clone 12	CATAGGCGATCGCCACCTATGTTTTAG	CACCAGTGCAACAGGAGCATAGTTAATATC

5′-CCACAGCATAGAGTTGGGCGAGTAA; *bicA* reverse, 5′ CAGAAATCA CGGTGTAGGGCATCAT; and *bicA* reverse nested, ATGGGCAATCACAG CGGTCATAA.

Luciferase assays and *luxAB* fusions. Putative promoter elements were amplified by PCR with the primers indicated in Table 4, cloned to pENTR (Invitrogen, Carlsbad, CA), and fused to *Vibrio* luciferase genes (*luxAB*) by Gateway LR recombination in a version of the *Synechococcus* sp. strain PCC 7002 neutral-site-integration reporter vector, pMBB, modified for lambda site-specific recombination. The pMBB vector (kindly provided by G. Bullerjahn) is a modified version of the *Synechococcus* sp. strain PCC 7942 luciferase reporter construct, pAM1414 (1), and contains a neutral integration site encoding omega-3 desaturase (*desB*) (21). Luminescence in liquid cyanobacterial cultures was measured in triplicate in freshly harvested 2-ml aliquots after the rapid addition of 4 μl of 0.025 to 0.05% (vol/vol) Decanal (D7384; Sigma, St. Louis, MO) in a TD-20/20 luminometer. Substrate was diluted in 100% dimethyl sulfoxide. The optimal decanal concentration was determined for each experiment. The average raw luminescence was normalized on a chlorophyll *a* basis and is expressed as a percentage of the empty vector control (high-C_i cells). The chlorophyll *a* concentration was determined as previously described (17).

Mass spectrometric measurements. Cells were prepared and analyzed by mass spectrometry as previously described (2, 25). Assays were performed in 4-ml volumes in a thermostat-controlled (30°C) mass spectrometer cuvette allowing membrane inlet analysis of O_2 (mass 32) and CO_2 (mass 44). For measurements of the photosynthetic affinity for C_i, cells were assayed at a chlorophyll density of 2 μg ml^{-1} in BG11 medium buffered with 50 mM BisTrisPropane-HCl (pH 7.9) in which NaNO$_3$ had been replaced with 20 mM NaCl. A light intensity of 700 μmol of photons m^{-2} s^{-1} was used. The maximum rate of net O_2 evolution (V_{max}) was measured in the presence of at least 1 mM NaHCO$_3$, and the photosynthetic affinity for C_i was determined as $K_{0.5}(C_i)$, that is, the C_i concentration required to reach half the maximum rate of net O_2 evolution. Measurements at low levels of C_i were initiated at around 25 μM O_2 and allowed to progressively increase throughout the C_i range.

RESULTS

Turnover of mRNA abundance for inducible C_i transporter genes. We have previously shown that transcripts encoding high-affinity C_i transport systems in *Synechococcus* sp. strain PCC 7002 are rapidly and strongly induced upon the transition to C_i limitation (20). To assess the importance of any transcriptional control of mRNA pool size, quantitative RT-PCR was used to monitor mRNA turnover in wild-type cells at high C_i or low C_i with or without the transcriptional inhibitor, RIF. The mRNA half-lives of *bicA* and *sbtA* (encoding inducible, high-affinity HCO$_3^-$ transporters), *chpY* (encoding part of an inducible, high-affinity CO_2 uptake system), and *porB* (encoding a putative HCO$_3^-$ porin) were measured (Fig. 1).

All transcript half-lives, estimated from the rate of mRNA degradation in the presence of RIF, were relatively short (approximately 5 to 10 min), irrespective of the C_i concentration. This suggests that any posttranscriptional control is not exerted differentially according to C_i concentration. Furthermore, cells

transferred from low C_i to high C_i in the absence of RIF had similarly rapid transcript turnover rates. That is, when transcriptional control in cells was maintained, transcripts were still equally rapidly turned over. Similar to previous findings in
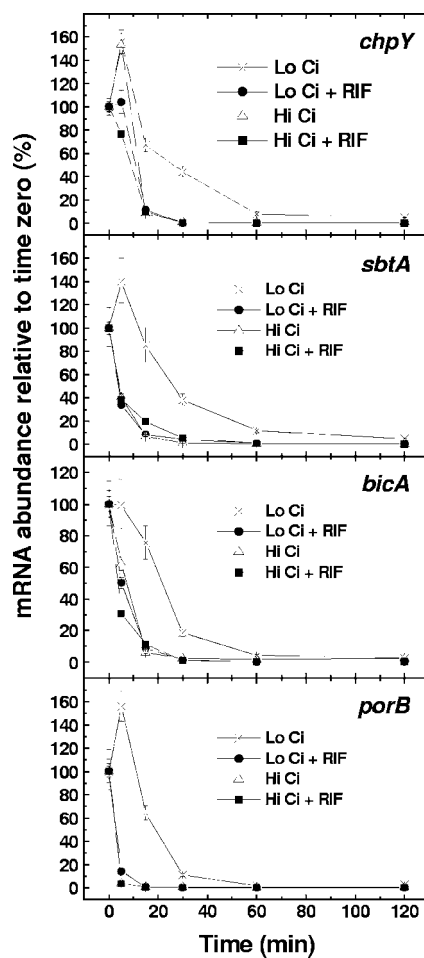

FIG. 1. Turnover of mRNA encoding C_i transporters in *Synechococcus* strain PCC 7002 cells. Exponentially growing high-C_i cells were swapped to buffer containing approximately 100 μM C_i and bubbled with CO_2-free air for 30 min. At this time (time zero) cells were transferred to either CO_2-free air or 2.0% CO_2 in the presence or absence of 200 μg of RIF ml^{-1}. The relative abundance of *chpY*, *sbtA*, *bicA*, and *porB* mRNA was determined by quantitative RT-PCR ($n = 4$). Symbols represent mRNA abundance relative to the 0-min amount (set at 100%) ± the standard error (SE).
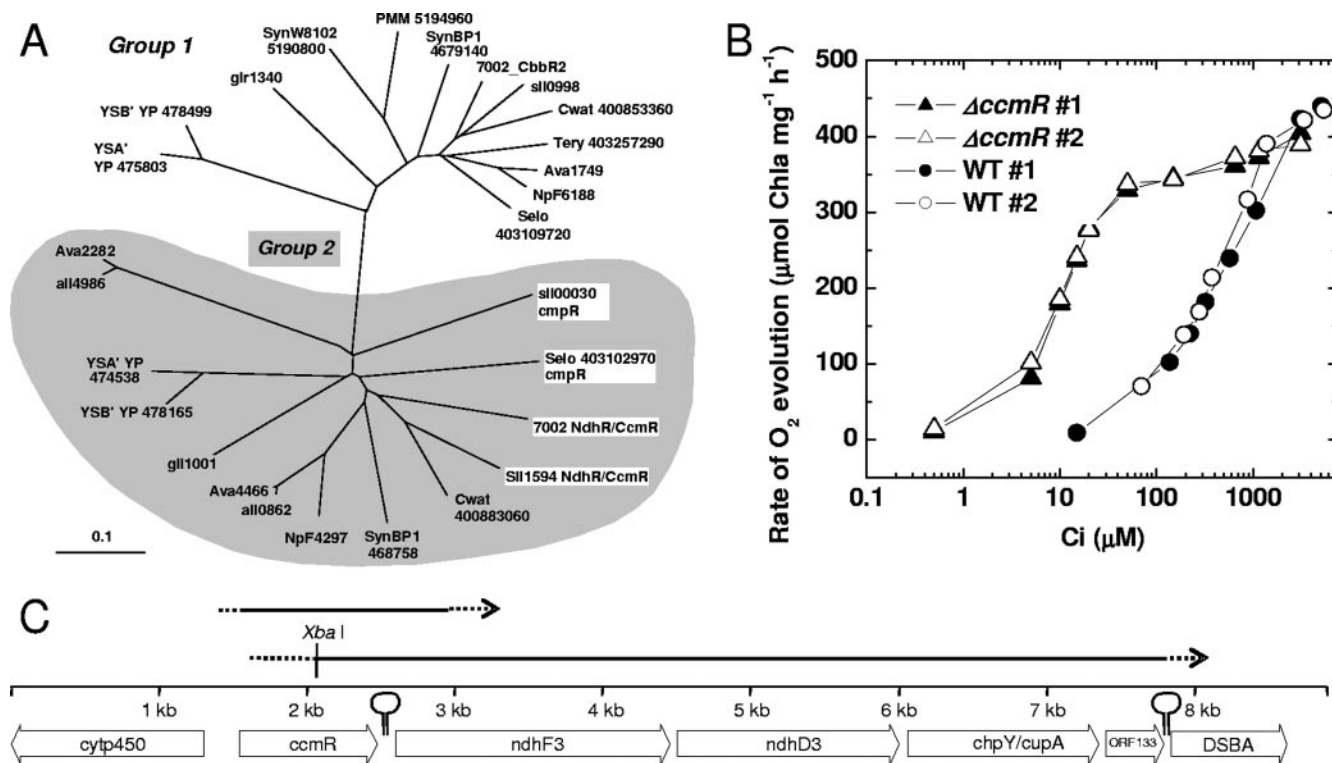
FIG. 2. CbbR-like genes in *Synechococcus* sp. strain PCC 7002. (A) Phylogenetic tree of proteins with >27% amino acid identity to *Rhodobacter sphaeroides* CbbR from cyanobacteria representing diverse habitats. Proteins were aligned by using CLUSTAL W and the dendrogram generated in the TREEVIEW program. Species name abbreviations: alr/all, *Nostoc* sp. strain PCC 7120; Avar, *Anabaena variabilis* ATCC 29413; Cwat, *Crocosphaera watsonii* WH8501; gll/glr, *Gloeobacter violaceus* (strain PCC 7421); Npun, *Nostoc punctiforme* ATCC 29133; PMM, *Prochlorococcus marinus* (subsp. *pastoris*, strain CCMP 1378/MED4); Selo, *Synechococcus* sp. strain PCC 7942 (elongatus); slr/sll, *Synechocystis* sp. strain PCC 6803; SynW8102, *Synechococcus* sp. strain WH8102; 7002, *Synechococcus* sp. strain PCC 7002; tlr/tll, *Thermosynechococcus elongatus* strain BP-1; Tery, *Trichodesmium erythraeum* IMS101; YSA′ and YSB′, *Synechococcus* sp. strain Yellowstone A-Prime and B-Prime. Locus tags are given for the *Anabaena*, *Synechocystis* strain PCC 6803, and *Gloeobacter violaceus* genes. GOIs from the Integrated Microbial Genomes database (DOE, Joint Genome Institute; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) are used elsewhere except for the Yellowstone species, for which the GenBank accession numbers are given. (B) Physiological phenotype of the group 2 "ccmR" deletion mutant. Rates of C_i-dependent O_2 evolution for wild-type (WT) and $\Delta ccmR$ cells grown continuously with 2% (vol/vol) CO_2 in air (two replicates are shown). (C) Structure of the *ccmR* genomic region in *Synechococcus* sp. strain PCC 7002. Possible cotranscribed regions are indicated with arrows (dashed lines represent the range of 5′ and 3′ delimits for each mRNA as indicated by RT-PCR; see Table 5). Predicted stem and loop structures are shown. *ndhF3/D3*, NAD(P)H-dehydrogenase (NDH-1_3) subunit genes; *chpY/cupA*, gene for putative CO_2 hydration protein associated with NDH-1; *orf133*, conserved gene of unknown function associated with NDH-1 genes; cytP450, cytochrome p450 (PFAM00067); DSBA, DSBA thioredoxin (PFAM 03123).

Synechococcus sp. strain PCC 7942 (29), these results suggest that control of the abundance of these transcript pools in response to C_i concentration is primarily transcriptional rather than posttranscriptional.

Deletion of a CbbR-like transcription factor (CcmR) derepresses CCM activity in *Synechococcus* sp. strain PCC 7002. We found that CbbR-like proteins from diverse cyanobacterial species cluster into two main groups (Fig. 2A). The less-divergent sequences (group 1) may represent proteins with an essential function, such as the Calvin cycle gene regulator CbbR, since this cluster contains the *Synechocystis* sp. strain PCC 6803 sll0998 locus which cannot be stably inactivated (7, 15, G. D. Price, unpublished data). A more divergent grouping (group 2) containing CmpR and NdhR/CcmR from *Synechocystis* sp. PCC 6803 (abbreviated as 6803) is evident. NdhR/CcmR_6803 would appear to repress the transcription of both *ndhF3 ndhD3 chpY* (*cupA*) and *sbtA* (7, 28), whereas CmpR_6803 appears to activate transcription of the *cmpABCD* operon (15). The freshwater strain *Synechococcus* sp. strain PCC 7942 has a single group

2 representative, CmpR_7942 (15). A single group 2 representative is also apparent in *Synechococcus* sp. strain PCC 7002.

We hypothesized that the single group 2 protein from *Synechococcus* sp. strain PCC 7002 might exert predominant control over the activity of the CCM. Although originally designated RbcR by Klughammer et al. (9), we tentatively renamed this factor CcmR (CcmR_7002), for the high-affinity CCM regulator, following the suggestion of Wang et al. (28). To test this idea, the CcmR_7002 ORF was deleted by transforming *Synechococcus* sp. strain PCC 7002 cells with a construct consisting of the flanking regions of *ccmR* fused to a kanamycin resistance marker. The segregation of the $\Delta ccmR$ allele from wild-type alleles after three rounds of kanamycin selection was confirmed by PCR (results not shown). As shown in Fig. 2B, mutant cells grown continuously at high C_i had a striking physiological phenotype. The cells have a very high photosynthetic affinity for C_i, with a $K_{0.5}(C_i)$ of approximately 10 μM, compared to the wild type, which has a $K_{0.5}(C_i)$ of approximately 250 μM. This result suggests that CcmR ordi-

narily functions to repress high-affinity CCM activity under nonlimiting C_i conditions.

CcmR represses expression of high-affinity C_i transporters in *Synechococcus* sp. strain PCC 7002 under nonlimiting C_i conditions. The expression of CcmR_7002 gene-flanking genes (Fig. 2C), high-affinity C_i transporter genes, and carboxysome-associated genes was assessed in $\Delta ccmR$ and wild-type cells bubbled with high C_i or air (Fig. 3). The mRNA abundance for the divergently oriented cytochrome p450 gene was largely unaffected by the replacement of the *ccmR* gene with a kanamycin resistance marker, irrespective of orientation (Fig. 3A). Thus, the mutant that contained the resistance marker in the same transcriptional orientation as *ccmR* was used in all subsequent experiments. The close juxtaposition in *Synechococcus* sp. strain PCC 7002 of *ccmR* and the genes for high-affinity CO_2 uptake—*ndhF3*, *ndhD3*, *chpY* (*cupA*), and *orf133*—is unique among the sequenced cyanobacterial genomes (Fig. 2C). Unlike the freshwater strain *Synechocystis* sp. strain PCC 6803 (7, 28), transcriptional derepression of this region was not observed in $\Delta ccmR$. Instead, a low-level constitutive expression of the region was found, irrespective of the C_i concentration or transgene orientation. It should be noted that the Tn*903* kanamycin cassette lacks a typical terminator and is likely to provide basal expression of the *ndhF3*, *ndhD3*, *chpY*, and *orf133* genes downstream of *ccmR* and be nonresponsive to CO_2. In contrast, and consistent with the increased photosynthetic affinity for C_i in the mutant (Fig. 2B), $\Delta ccmR$ cells grown at high C_i had relatively abundant mRNA pools for the inducible HCO_3^- transporter genes *bicA* and *sbtA* and the putative HCO_3^- porin gene, *porB*, compared to wild-type cells (Fig. 3B). These levels approached the maximal levels in air-induced wild-type cells (Fig. 3B). We also investigated whether carboxysome-associated genes are targets for CcmR action but found little difference in the mRNA abundance for these genes between mutant and wild-type cells (Fig. 3D). Given that these genes are not strongly C_i responsive in the wild type, this result is not surprising. An exception was the mRNA pool encoding the carboxysomal CA, *ccaA*, which was more strongly induced under air limitation in $\Delta ccmR$ cells. This mild induction of *ccaA* mRNA has been noted in other CCM-deficient mutants (6).

Transcriptional regulation of the CcmR region in *Synechococcus* sp. strain PCC 7002. The proximity of the CcmR_7002 gene to the *ndhF3 ndhD3 chpY* (*cupA*) *orf133* gene cluster (Fig. 2C), plus the lack of transcriptional C_i responsiveness of this region in the $\Delta ccmR$ mutant (Fig. 3B), led us to investigate the transcriptional regulation of this region. We generated an insertional *ccmR* mutant at the XbaI site (Fig. 2C) located 420 bp from the start of the *ccmR* ORF; however, the phenotype was the same as the deletion mutant (results not shown). This result indicates that it is difficult to engineer mutations at this locus that do not interfere with transcription of the downstream gene cluster. The expression of the 5′ half of the *ccmR* transcript, upstream of the insertion site for the antibiotic resistance marker, was monitored in the insertional mutant by quantitative RT-PCR and found to be elevated at high C_i (Fig. 4B). This result signifies that CcmR ordinarily autorepresses *ccmR* transcription, as previously found for NdhR/CcmR in the freshwater strain *Synechocystis* sp. strain PCC 6803 (7).

An RT-PCR and quantitative RT-PCR approach was taken to define the limits of the mRNA(s) which encompasses *ccmR*
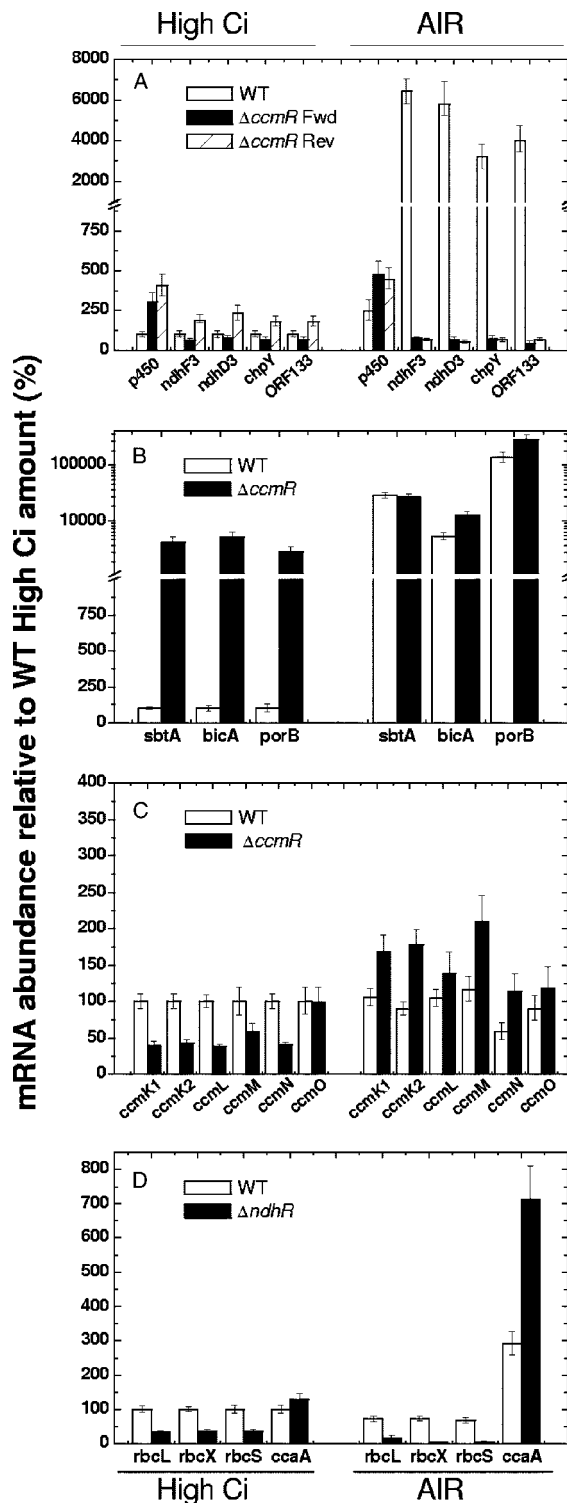

FIG. 3. Gene expression in $\Delta ccmR$. The relative abundance of mRNA for *ccmR*-neighboring genes (A), bicarbonate transporter genes (B), and carboxysomal genes (C and D) in wild-type and $\Delta ccmR$ cells after transfer from bubbling with 2% (vol/vol) CO_2 in air to bubbling with air alone for 60 min was determined. Transcript changes were determined by quantitative RT-PCR ($n = 4$), and symbols represent transcript expression relative to the wild-type (WT) 0-min amount (set at 100%) $\pm$ the SE. Note the break in the y axis in panels A and B. The experiment was independently replicated, and representative data are shown.

and the downstream ORFs [*ndhF3*, *ndhD3*, *chpY* (*cupA*), and *orf133*]. The transcription of the entire region was coinduced in response to Ci limitation in wild-type *Synechococcus* sp. strain PCC 7002 cells (Fig. 4A); however, only very weak transcription of the sequence downstream from *orf133* was detected. The results of RT-PCR, using first-strand cDNA generated from the set of reverse primers described in Tables 1, 2, and 3 and primer pairs that span the junctions of the ORFs in this region of the genome (Table 1), are summarized in Table 5. As previously speculated (9), the *ndhF3*, *ndhD3*, *chpY* (*cupA*), and *orf133* ORFs probably form part of an operon, since it was possible to amplify at least 250 bp beyond the junctions of all of these ORFs. It was also possible to amplify significantly overlapping fragments of the *ccmR* and *ndhF3* ORFs from first-stand cDNA, although based solely on this analysis the two ORFs are not necessarily part of the same mRNA (see Table 5, the 1521F-2876R and 1521F-3215R primer pairs).

An analysis of the region upstream of the start codon of *ndhF3* revealed putative −35 and −10 elements and LysR binding sites (TnA{n7/8}TnA; Fig. 4C). Using RLM-RACE, a TSP was mapped to a point that supports the predicted promoter structure (Fig. 4C). To determine whether the region 5′ to *ndhF3* can function as a promoter, the 550 bp upstream of the *ndhF3* start codon was fused to genes encoding *Vibrio* luciferase (*luxAB*) in a neutral-site integration reporter vector, pMBB. However, no significant luciferase activity was detected in wild-type cells transformed with the resulting construct at high Ci or after 5 h of air bubbling (Fig. 4D), suggesting that this region does not contain a functional promoter.

The *bicA* gene is part of a large CcmR-regulated operon. Our finding that *Synechococcus* sp. strain PCC 7002 CcmR represses full expression of the *bicA* gene at nonlimiting Ci (Fig. 3B) led us to examine the regulation and function of neighboring genes. Figure 5A shows the genomic organization of the region surrounding the *bicA* locus (GenBank accession no. AF381039). Suggestive of the existence of an operon, nine ORFs occur immediately downstream of *bicA* without significant intergenic sequences. Immediately downstream of *bicA* is an ORF that encodes a protein with domains of a Na⁺/H⁺ antiporter from the CPA1 family, HMM PF00999, which we have termed *nhaS3* after the *Synechocystis* sp. strain PCC 6803 homologue (sll0689). The subsequent eight ORFs encode hydrophobic sequences resembling a class of cation/proton antiporter proposed to function as a hetero-oligomer (27). The nomenclature of these transporters is unresolved. We have chosen Mnh for multisubunit Na⁺/H⁺ antiporter (alternatively Mrp, Mnh, Pha, Sha 27), since *Nostoc* strain PCC 7120 mutants at a similar locus display Na⁺ sensitivity (5). Several of these Mnh genes in *Synechococcus* sp. strain PCC 7002 encode proteins with similarity to the hydrophobic subunits of proton-pumping NADH:ubiquinone oxidoreductases, including MnhC (HMM PF00420), MnhD1/2 (HMM PF00361), and MnhB (HMM PF04039); however, their general role, if any, in transporter energization is obscure (27). This tripartite clustering of genes encoding transporters—*bicA*, *nhaS3*, and *mnh*—is unique within the sequenced cyanobacterial genomes.

Quantitative RT-PCR analysis (Fig. 5B) showed that *nhaS3* plus the *mnh*-like genes in *Synechococcus* sp. strain PCC 7002 are upregulated in response to Ci limitation and, as with *bicA*, *nhaS3* and *mnh* gene expression is also derepressed at high Ci in

Δ*ccmR* (Fig. 5C). Previously, similar regulation of *mnh* genes in the freshwater strains *Synechocystis* sp. strain PCC 6803 (28) and *Nostoc* sp. strain PCC 7120 (5) has been demonstrated. RT-PCR was carried out with primers that span the junctions of the ORFs in the *bicA* region in *Synechococcus* sp. strain PCC 7002 (Table 2). Consistent with the cotranscription of these genes as an operon (Table 6), products that span the intergenic regions were successfully amplified from first-strand cDNA. The failure to detect products at 300 bp upstream of the presumptive *bicA* start codon suggests the 5′ untranslated region is contained in the intervening region (Table 6, primer pair 1279F-1980R). Relatively weak, Ci-responsive transcription was detected up to 172 bp beyond the presumptive stop codon for MnhB (Fig. 5A); however, quantitative RT-PCR showed that, beyond this point, transcripts were not detectable above background levels (results not shown), even though very weak bands were detected using a nonquantitative RT-PCR endpoint approach (Table 6, primer pair 10181F-10692R).

The *bicA* operon is essential for full bicarbonate transport activity. To assess the potential role of the *nhaS3 mnh* gene cluster in HCO₃⁻ uptake, the first three ORFs in this region were inactivated by engineering a deletion between the SmaI and XbaI sites noted in Fig. 5A. This strategy was adopted given the possibility that this gene cluster encodes redundant functions. Segregation of the resulting Δ*nha* allele from wild-type alleles was confirmed by PCR after three rounds of chloramphenicol selection (results not shown). The HCO₃⁻-dependent oxygen evolution (a surrogate measure for net HCO₃⁻ uptake) was determined in air-grown wild-type and Δ*nha* cells incubated with the known CO₂ uptake inhibitor, ethoxyzolamide (EZ) (18, 20). Control cells were incubated with 10 μg of bovine CA/ml to ensure rapid equilibrium of Ci species. As shown in Fig. 5D, at Ci concentrations between approximately 20 and 130 μM, oxygen evolution in mutant cells incubated with 300 μM EZ was markedly reduced compared to the wild type. That is, when active HCO₃⁻ uptake was the major route for Ci uptake, oxygen evolution was diminished in the mutant but not in the wild type. The relative photosynthetic affinity for Ci in the presence of EZ, as measured by the $K_{0.5}(C_i)$, was 15.5 ± 2.6 μM in the wild type but only 74.9 ± 1.5 μM in the mutant (Fig. 5E). This suggests that the *nhaS3-mnh* region ordinarily has a role in supporting maximal HCO₃⁻ uptake in this cyanobacterium. The growth of the mutant was investigated at high pH, under air limitation, to maximize the contribution of HCO₃⁻ transport to growth. However, no difference in growth rate compared to the wild type was detected (data not shown).

Analysis of the structure of the *bicA* promoter. To identify potential Ci-responsive elements within the region immediately upstream and downstream of the *bicA* start codon, a series of DNA fragments from this region (Fig. 6A) were fused to genes encoding *Vibrio* luciferase (*luxAB*) in a version of the *Synechococcus* sp. strain PCC 7002 neutral-site integration reporter vector, pMBB, modified for lambda site-specific recombination. Elements were selected according to proximity to consensus LysR binding sites and predicted −35 and −10 elements (Fig. 6B). Using RLM-RACE, several TSPs were mapped to a region that supports this predicted promoter structure (Fig. 6B).

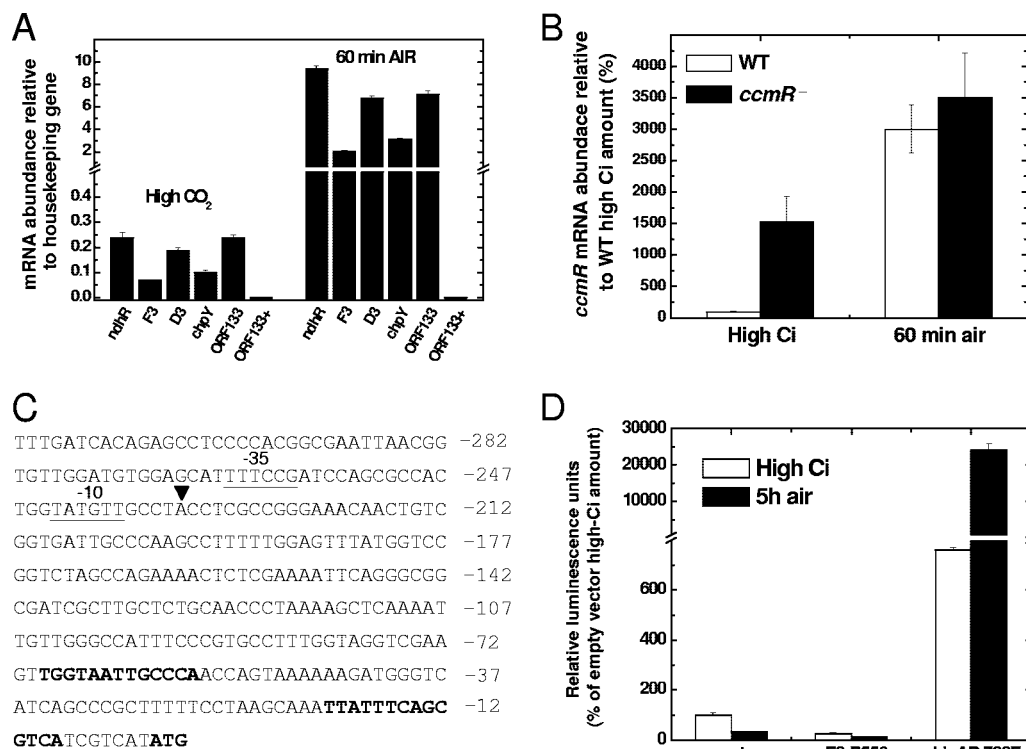The Ci-responsive luciferase activity of *bicA* promoter-*luxAB*

FIG. 4. Transcriptional regulation of the *ccmR* region in *Synechococcus* strain PCC 7002. (A) Relative mRNA abundance of the ORFs downstream from *ccmR* in wild-type cells transferred from bubbling with 2% CO_2 in air to bubbling with normal air. Transcript changes were determined by quantitative RT-PCR ($n = 4$), and symbols represent transcript expression ± the SE relative to the housekeeping gene, *rnpA* (set at 1.0). (B) Relative mRNA abundance of *ccmR* in wild-type (WT) or *ccmR* insertional mutant cells after transfer from bubbling with 2% (vol/vol) CO_2 in air to bubbling with normal air. Transcript changes were determined by quantitative RT-PCR ($n = 4$), and symbols represent transcript expression relative to the wild-type high-C_i amount (set at 100%) ± the SE. Note the break in the *y* axis in panel A. (C) Predicted promoter structure of the *ndhF3* upstream region. Consensus LysR binding sites are in boldface, and putative −35 and −10 elements (as predicted by BPROM) are also shown. The most 5′ TSP determined experimentally is marked with an arrow. (D) Luciferase activity directed by the 550-bp *ndhF3* upstream region fused to *luxAB* genes (pF3:P550) and stably integrated into the genome of *Synechococcus* sp. strain PCC 7002 cells. Cells were grown at high C_i or with air bubbling for 5 h. A positive C_i-responsive control (pbicA:P722F) consisting of a 722-bp fragment from the upstream region of the *bicA* gene fused to luxAB, is shown (see Fig. 6A). Relative luminescence units are expressed on a chlorophyll *a* basis as a percentage of the value obtained for high-C_i cells containing the empty vector control ± the standard deviation.

transformants was measured (Fig. 6A). The region approximately 500 nucleotides upstream and 200 nucleotides downstream of the *bicA* start codon directed low-C_i responsive luciferase activity. The reverse orientation of this same element produced a low, constitutive luciferase activity, perhaps from elements contained in the divergently oriented promoter region from the neighboring transglycosylase gene (Fig. 5A). A series of 5′ and 3′ truncations (clones 2, 4, 5, 7, 8, and 12) suggest that the basal promoter sequences and C_i response elements are contained between positions −95 and −239 (clone 12) relative to the *bicA* start codon (note the difference in activity between clone 12 and clone 8, Fig. 6A). This small region directs inducible luciferase activity at low C_i but not to the same degree as the longest region investigated (−515 to +208), which would appear to contain enhancer elements (compare clone 722F with clone 12). Interestingly, the deletion

TABLE 5. Amplification of mRNA sequences from the *ccmR* region of *Synechococcus* strain PCC 7002 by RT-PCR[a]

Primer pair coordinates	Junction	Product	Primer pair coordinates	Junction	Product[b]
1350F-2002R	Upstream/*ccmR*	N	4228F-4670R	*ndhF3*/*ndhD3*	Y
1521F-2002R	*ccmR*/*ndhF3*	Y	5806F-6253R	*ndhD3*/*chpY*	Y
1521F-2876R	*ccmR*/*ndhF3*	Y	7172F-7602R	*chpY*/ORF133	Y
1521F-3215R	*ccmR*/*ndhF3*	N	7589F-7963R	ORF133/downstream	Y*
1521F-4670R	Upstream/F3	N	7589F-8288R	ORF133/downstream	Y*
1950F-4670R	Upstream/F3	Y	82737F-8726R	ORF133/downstream	Weak*

[a] The sequence map coordinates of primers (according to Fig. 4; GenBank accession no. U97516) and the presence (Y) or absence (N) of an amplified product are indicated. First-strand cDNA template was generated from RNA derived from *Synechococcus* strain PCC 7002 cells transferred from 2% CO_2 to air bubbling for 30 min.
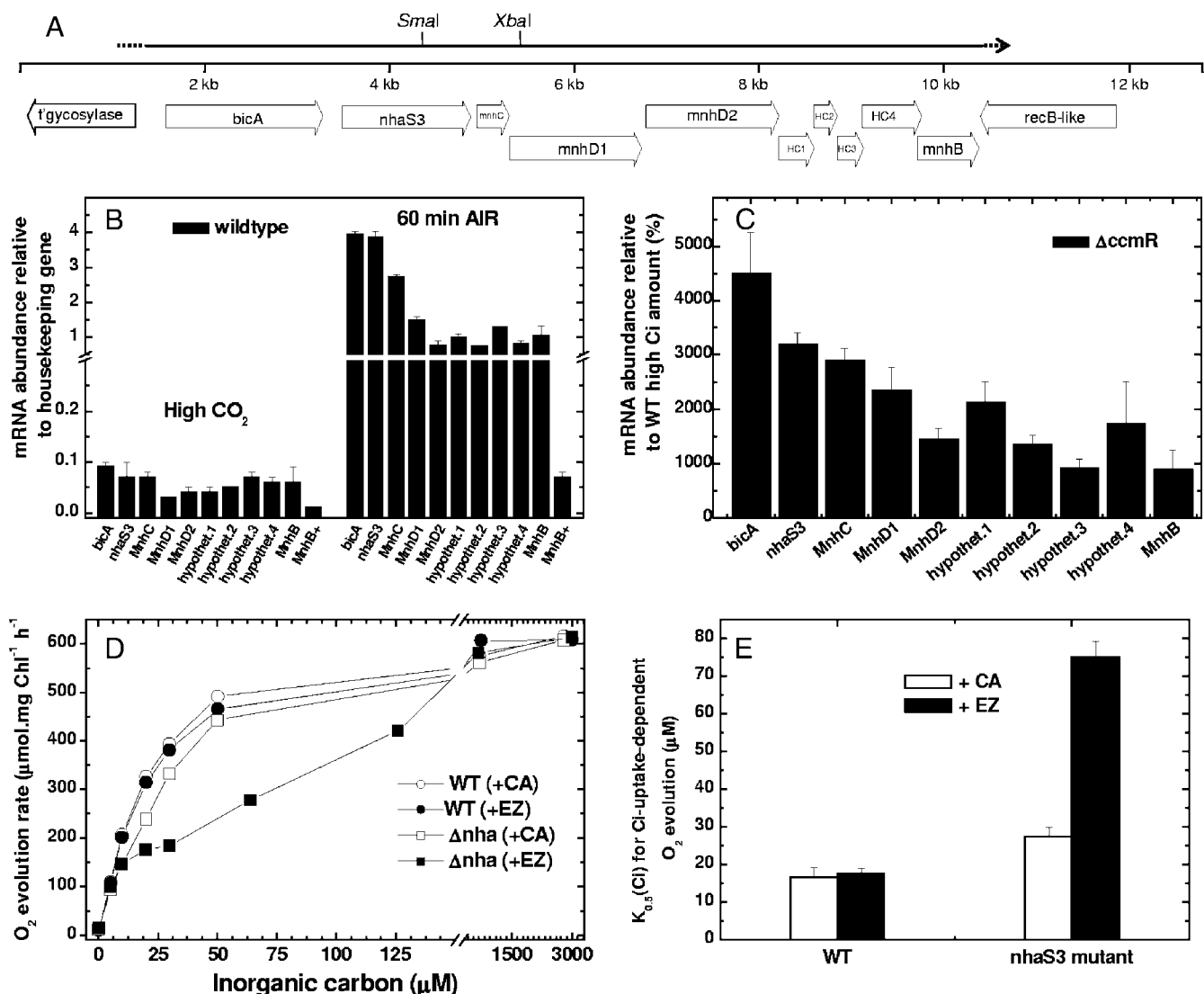
[b] *, Not C_i responsive.

FIG. 5. Structure, regulation, and function of the *bicA* genomic region in *Synechococcus* strain PCC 7002. (A) Map of the *bicA* genomic region. Cotranscribed regions are indicated with arrows (dashed lines represent the range of 5′ and 3′ delimits for each mRNA as determined by RT-PCR; see Table 6). nhaS3, Na$^+$/H$^+$ antiporter (HMM PF00999); *mnhC* (HMM PF00420), *mnhD1/2* (HMM PF00361), and *mnhB* (HMM PF04039), NADH:ubiquinone oxidoreductase-like subunits from a putative multisubunit Na$^+$/H$^+$ antiporter; T'glycosylase, transglycosylase (PFAM03562); *recB*, nuclease (COG2251.1); HC, hypothetical conserved protein. (B) Relative mRNA abundance of the ORFs downstream of *bicA* in wild-type (WT) cells transferred from bubbling with 2% CO_2 in air to bubbling with air. Transcript changes were determined by quantitative RT-PCR ($n = 4$), and symbols represent transcript expression ± the SE relative to the housekeeping gene, *rnpA* (set at 1.0). (C) Relative mRNA abundance of ORFs in the *bicA* region in wild-type (WT) or $\Delta ccmR$ mutant cells bubbled continuously with 2% (vol/vol) CO_2 in air. Transcript changes were determined by quantitative RT-PCR ($n = 4$), and symbols represent transcript expression relative to the wild-type 0-min amount (set at 100%) ± the SE. (D) HCO_3^--dependent oxygen evolution in air-grown wild-type cells and cells containing a deletion between the indicated SmaI and XbaI sites (see panel A) of the *nhaS3* and *mnhD1* genes (Δnha). Cells were analyzed by membrane inlet mass spectrometry at the indicated C_i concentrations in the presence of added CA or the CO_2 uptake inhibitor, EZ. (E) Relative photosynthetic affinity for C_i for cells (part D) as measured by the $K_{0.5}(C_i)$ (C_i concentration for half maximal rate). Note the break in the y axis in panel B.

of two putative LysR binding sites from the 5′ and 3′ regions of clone 12 (yielding clone 10) resulted in an apparent inversion in the C_i responsiveness of this region. That is, clone 10 produced strongly derepressed luciferase activity at high C_i (to levels exceeding those of air-induced clone 12), but this activity was apparently suppressed under C_i limitation (Fig. 6A). However, the luciferase activity in the negative controls (empty vector and 722R) was also repressed to a similar degree at low C_i, suggesting the existence of a generalized repression of

luciferase activity under this stress condition. We ascribe this to a general downturn in protein synthesis under C_i limitation.

DISCUSSION

We investigated the organization and regulation of CO_2-concentrating mechanism genes in a euryhaline, coastal/estuarine cyanobacterium, *Synechococcus* sp. strain PCC 7002. It was found that mRNA abundance for high-affinity C_i transporter genes (20)

TABLE 6. Amplification of mRNA sequences from the *bicA* region of *Synechococcus* strain PCC 7002 by RT-PCR[a]

Primer pair coordinates	Junction	Product	Primer pair coordinates	Region or junction	Product
1279F-1980R	Upstream/*bicA*	N	8014F-8486R	ORF5/6	Y
1462F-1980R	Upstream/*bicA*	Y	8333F-8774R	ORF6/7	Y
1561F-1980R	Upstream/*bicA*	Y	8613F-9081R	ORF7/8	Y
3198F-3700R	*bicA*/*napA*	Y	8923F-9325R	ORF8/9	Y
4697F-5247R	*napA*/ORF3	Y	9510F-9976R	ORF9/10	Y*
5061F-5462R	ORF3/4	Y	10140F-10539R	ORF10/downstream	Y*
6718F-7128R	ORF4/5	Y	10181F-10692R	ORF10/downstream	Med*

[a] The sequence map coordinates of primers (according to Fig. 5A; GenBank accession no. AF381039) and the presence (Y) of an amplified product are indicated. First-strand cDNA template was generated from RNA derived from *Synechococcus* strain PCC 7002 cells transferred from 2% CO_2 to air bubbling for 30 min. *, Not C_i responsive.

is controlled primarily at the transcriptional level (Fig. 1). We inactivated a gene encoding a LysR transcription factor that resembles the proteobacterial Calvin cycle regulator CbbR and which in phylogenetic analyses is found in a divergent grouping of cyanobacterial CbbR-like factors containing the NdhR/CcmR and CmpR factors from freshwater cyanobacteria (group 2; Fig. 2A). Consistent with an elevated mRNA abundance for medium and high-affinity HCO_3^- transporter systems (Fig. 3), the mutant strains grown at high C_i had fully induced CCMs at a physiological level (Fig. 2B). Accordingly, since the gene product regulates the transition from a basal CCM to a CCM with high affinity for C_i, we renamed this gene *ccmR*, for CCM regulator as suggested by Wang et al. (28). We also found that CcmR_7002 regulates a unique 10-gene operon containing the low-affinity HCO_3^- trans-

porter gene, *bicA*, and genes for two likely Na^+/H^+ antiporters that are essential for full HCO_3^- uptake activity (Fig. 5). The minimal *bicA* promoter was defined, along with C_i response elements contiguous with consensus LysR binding sites (Fig. 6).

Prior to the present study, little physiological data has been reported for LysR regulators of CCM genes (NdhR/CcmR and CmpR), and there is an apparent complexity of function within the group 2 factors (i.e., both activators and repressors; Fig. 2A). The striking physiological phenotype of the *Synechococcus* sp. strain PCC 7002 group 2 Δ*ccmR* mutant, in which CCM activity was fully induced at high C_i (reported here for the first time in cyanobacteria), was consistent with the types of genes that were shown to be constitutively upregulated (Fig. 3). Identified gene targets of CcmR repressor function include the
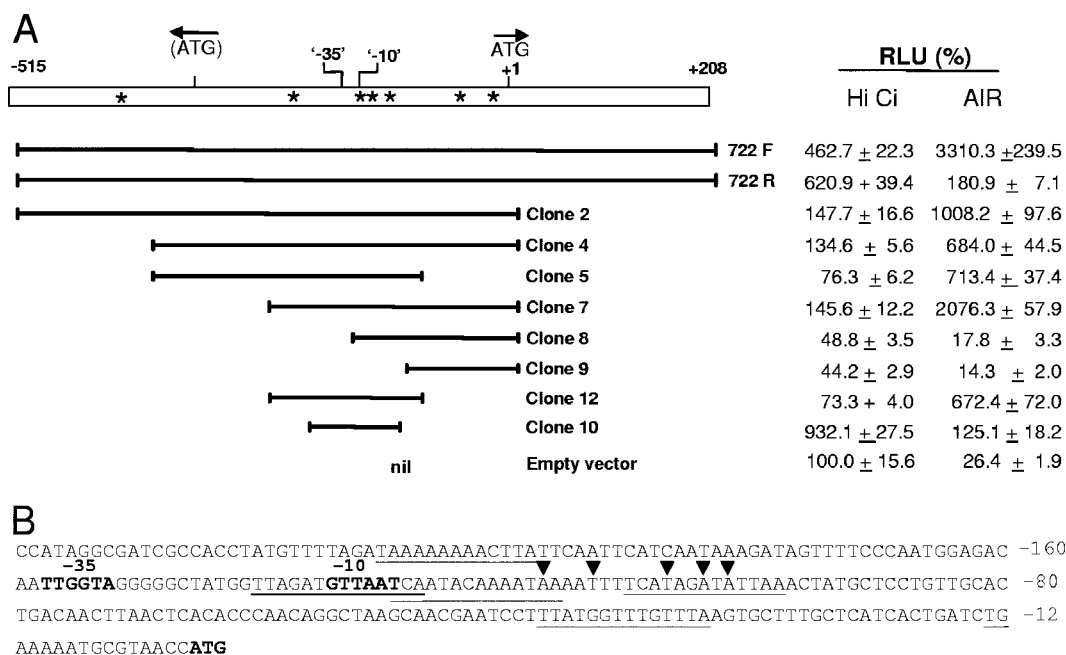

FIG. 6. Analysis of the *bicA* promoter region in *Synechococcus* sp. strain PCC 7002. (A) Luciferase activity directed by *bicA* upstream elements fused to *luxAB* genes and stably integrated into the genome of *Synechococcus* sp. strain PCC 7002 cells. Cells were grown at high C_i or with air bubbling for 5 h. Relative luminescence units are expressed on a chlorophyll *a* basis as a percentage of the value obtained for high-C_i cells containing the empty vector control ± the standard deviation. Asterisks indicate the location of putative LysR-binding motifs. (B) Predicted promoter structure of the *bicA* upstream region in *Synechococcus* sp. strain PCC 7002. Putative LysR binding sites are underlined, and putative −35 and −10 elements (as predicted by BPROM at www.softberry.com) are shown in boldface. The TSPs detected by using RLM-RACE are marked with arrowheads; although it is usual to place functional bias on the longest start points relative to the start codon, the shorter products may be genuine alternative TSPs. Note that the first base of the putative −10 box is a G and not the more usual T.

A. *sbtA*

```
                                    -35                    -10        ▼
CCTTAACTAGGAGCAATGAAGACAATAGAAAAGAAAAACTTATGAATTAAATTTGACATTGCGTATCAAATCCAGGCTG -199
AAAAACGTAAAAATCTGCGCAATTCAAGATAGACTTAAGCACATAGAAATTTGGGTGTTAATAGAGCTAAATCTATAAA -119
AGCCCGATAACAGATTTTCGCATCGTACCCGCGCTGTAGGTGTGAAGCCCTGCAGCCTTACTATTCAAAGCTCACAATT  -39
ACCGCTCTTCATGGGAGGAAGCGGAAGGAGGTATTAGTG
```

B. *porB*

```
                  -35                      -10          ▼
ACAGAAACAATTGGAACTTTCTTGGGCATTTTAAACAATTTTATATTTATCGAGGAGGAATCTACTGTTATG +3
```

C. *ccmR*

```
                          35                        10          ▼
AACCGAAACTATGACGATATTTTCTTGTATTGACTCTAGTCTATGGTATTGCTAAACTCAGGCATAACAGCACAAGTTC -159
AGGAGTAAACCCTTGATCCCTGCAACTCTCCACCAACTGAAGGTGTTTGAAACCACGGCTCGCCACGGCAGTTTTACCA  -76
AGGCAGCGGAGGAATTATCAATCACCCAACCGACGGTTTCTGGACAAGTGAAACAACTGACCCAGACCATCGGGATG    +3
```
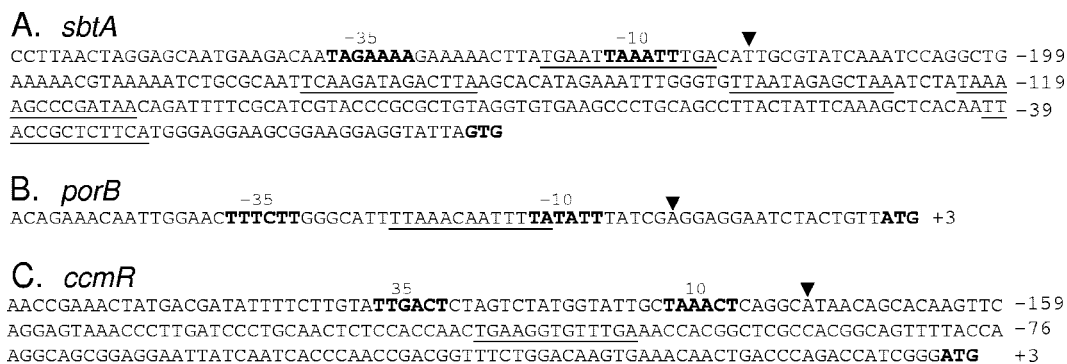
FIG. 7. Putative promoter structure of the *sbtA* (A), *porB* (B), and *ccmR* (C) upstream regions in *Synechococcus* sp. strain PCC 7002. Putative LysR binding sites are underlined, −35 and −10 elements are in boldface, and predicted TSPs are marked with an arrowhead (as predicted by BPROM).

known loci for high-affinity HCO_3^- transporters, *sbtA* and *bicA*, as well as that for the presumptive HCO_3^- porin, *porB*. Putative LysR binding motifs (TnA{n7/8}TnA, 22) exist within 500 bp of the *sbtA*, *porB*, and *bicA* start codons in the *Synechococcus* sp. strain PCC 7002 genome (Fig. 6 and 7). Interestingly, *bicA* was not previously identified as a target for NdhR/CcmR in *Synechocystis* sp. strain PCC 6803 on microarrays (28), since the gene appears to be constitutively expressed in this strain. Indeed, there is no recognizable *bicA*-like gene in the genome of the much-studied *Synechococcus* sp. strain PCC 7942.

Owing to the parallel orientation and proximate location of *ccmR*, we were unable to confirm whether the *ndhF3 ndhD3 chpY* (*cupA*) *orf133* operon is also a target for CcmR repression, as was found for CcmR_6803 (7, 28). However, LysR factors are often located in proximity to target genes (22), and a tight relationship exists in cyanobacterial genomes between the presence of a *ccmR*-like gene and specific genes of the NDH-1₃ complex (see below). These observations are highly suggestive of a functional relationship. From the present study it is unclear whether *ccmR* is expressed as a monocistronic mRNA or whether this gene is cotranscribed from a single promoter upstream of *ccmR* together with the *ndhF3*, *ndhD3*, *chpY* (*cupA*), and *orf133* genes under some or all growth conditions. The latter option (cotranscription) is supported by our finding that the region 550 bp upstream of the start codon of *ndhF3* does not encode a functional promoter under standard growth conditions (Fig. 4D) and also that there were similar levels of mRNA detected for each ORF in this region at both high C_i and low C_i (Fig. 4A). That mRNA for CcmR (a repressor) may be cotranscribed with part of the CcmR regulon is counterintuitive. LysR repressors are commonly transcribed divergently from neighboring target genes, as is the case for CcmR (NdhR) and Slr1727 in *Synechocystis* sp. strain PCC 6803 (7). Significantly, a predicted stem-loop structure is found within the *ccmR-ndhF3* intergenic region (Fig. 2C). It is possible that this structure is a target for a regulatory mechanism to produce nonstoichiometric levels of mRNA or protein from this region, for example, via antitermination factors for RNA polymerase, or masking of ribosome entry sites. Alternatively, posttranslational regulation of CcmR by a C_i-related signal such as cytosolic HCO_3^-, production of which is directly linked to the physiological activity of the associated *ndhF3* regulon,

might constitute a sufficient mechanism to ensure adequate modulation of mRNA for the high-affinity CO_2 uptake system. Certainly, mRNA for *ccmR* and high-affinity C_i uptake systems is initially strongly induced but returns rapidly (60 to 90 min) to a low, constitutive level after C_i depletion (20). In addition, studies with the freshwater strain *Synechococcus* sp. strain PCC 7942 suggest that proteins encoding the high-affinity C_i uptake systems are only very slowly turned over (29). Hence, functional transporters could persist after an initial burst of transcription. More work is required to resolve this question.

Our finding that *bicA* is a target for CcmR function in *Synechococcus* sp. strain PCC 7002 prompted a closer examination of the regulation and function of the *bicA* genomic region (Fig. 5). An operon of 10 ORFs was defined: it commences with *bicA* and encodes two likely Na^+/H^+ antiporters, a CPA1 family member similar to NhaS3 from *Synechocystis* sp. strain PCC 6803 and a multigene Na^+/H^+ transporter that we have termed Mnh with components resembling NADH:ubiquinone oxidoreductases. This tripartite clustering of transporter activities is unique among known cyanobacterial genomes; however, some clustering of *bicA*-like genes with likely Na^+/H^+ antiporter genes can be discerned in other organisms, including *Crocosphaera watsonii* WH8501 (GOI 400892570) and *Nostoc* sp. strain PCC 7120 (GOI 4222050). Our deletion of a region downstream of *bicA* showed that it encodes factors essential to full HCO_3^- utilization (Fig. 5D). Although a C_i-responsive *mnh*-like operon is known in both *Synechocystis* sp. strain PCC 6803 and *Nostoc* sp. strain PCC 7120 (5, 28) and implicated in salt stress in *Nostoc* sp. strain PCC 7120, the potential role of the complex, if any, in HCO_3^- uptake was uncertain: the Mnh complex may be a primary extrusion mechanism to maintain the Na^+ gradient required for HCO_3^- uptake, or it may harness an existing proton gradient to mitigate the cytosolic alkalinization resulting from HCO_3^- uptake (the subsequent conversion to and fixing of CO_2 would necessitate effective proton exchange systems). This might be particularly significant in the case of BicA, which sustains a relatively high flux rate compared to other inducible systems (20). Our new data (Fig. 5D and E) showing that the genes from the *bicA* operon are required for maximal HCO_3^--uptake activity are consistent with both ideas, and further work is needed to more fully understand the physiological functions encoded by this operon.

We examined the structure of the *bicA* promoter by fusing a series of upstream elements to genes encoding *vibrio* luciferase and by measuring the resultant C_i-responsive luciferase activity of transformants. We identified a minimal 144-bp region required for C_i-responsive expression (Fig. 6A), as well as two LysR consensus-binding sites flanking the presumptive −35 and −10 elements that, when deleted, result in derepression of the promoter at high C_i. We speculate that, at high C_i, one or more of these sites ordinarily binds CcmR, suppressing futile expression of the operon, not unlike the situation for control of the *ndhF3* promoter in *Synechocystis* sp. strain PCC 6803 (7). We are currently exploring the nature of any physical interaction between these elements and CcmR_7002 given the strongly positive evidence obtained in the present study that there is a functional link between CcmR and *bicA* regulation.

Our survey of CbbR-like factors in cyanobacteria revealed several interesting relationships (Fig. 2). All genomes investigated encoded a single CbbR-like protein belonging to a highly conserved family (group 1), which may indicate an essential function such as Calvin cycle gene regulation. Consistent with this idea, group 1 contains the *Synechocystis* sp. strain PCC 6803 sll0998 locus, which cannot be stably inactivated (7, 15; Price, unpublished). Based on Rubisco subtype (4), and exclusively open-ocean dwelling, the "alpha" strains are not represented in the more divergent group 2. With the exception of *Trichodesmium erythraeum*, the "beta" strains (freshwater species and some coastal or offshore marine strains) contain one or two group 2 proteins. The group 2 genomes also encode recognizable NDH-1_3 complexes for high-affinity CO_2 transport (4) and, with the exception of *Gloeobacter violaceus*, all have two or three likely high-affinity HCO_3^- transporters (4). Conversely, strains that do not contain genes for group 2 proteins lack the genes for a recognizable NDH-1_3 complex, including *Trichodesmium erythraeum*. Owing to a habitat typified by fluctuating C_i concentrations, we speculate that group 2 CbbR-like proteins are a habitat-specific adaptation that is related to a requirement for high-affinity CO_2 transport. Intriguingly, *Synechococcus* sp. strain PCC 7942 has no recognizable CcmR-like protein and yet it possess functional transporters encoded by *cmpABCD*, *sbtA*, and *ndhF3 ndhD3 chpY* (*cupA*) (19). Instead, it contains a single group 2 protein, CmpR, which is believed to function as an activator of the *cmp* operon, which encodes a high-affinity HCO_3^- transporter (15). This raises questions about how the CCM is regulated in *Synechococcus* sp. strain PCC 7942.

In conclusion, we have shown the importance of a "derepression" mechanism for CCM regulation in a coastal/estuarine cyanobacterium. Under growth conditions in which C_i is not limiting, a single LysR transcription factor, CcmR, is essential for strong and coordinate transcriptional repression of multiple genes encoding high-affinity C_i transporters. We hypothesize that under limiting C_i, CcmR is transiently inactivated, even though the mRNA for this factor is still abundant. Further work in this species could profitably focus on the role of metabolite binding (possibly HCO_3^-) in the regulation of CcmR activity. Our survey of CbbR-like genes in cyanobacterial genomes shows that related genes are present only in the beta cyanobacteria that likely use high-affinity CO_2 transport systems. Consequently, we propose that CcmR is a habitat-specific specialization for species that exist in environments subject to widely fluctuating C_i concentrations. Given the divergence of the NdhR/CmpR subgroup of LysR factors in the beta cyanobacteria and the fact that *Synechococcus* sp. strain PCC 7942 encodes only a more distant form of CcmR, a species-specific approach to characterizing CCM regulation is warranted. Considering the long evolutionary history of the cyanobacteria, it should not be surprising that organisms from differing ecological niches have evolved independent solutions to the problem of optimizing their C_i acquisition systems. The differences we have uncovered in the present study between *Synechococcus* sp. strain PCC 7002 and the freshwater models are a case in point.

ADDENDUM IN PROOF

Details of the construction of the pMBB reporter plasmid can be found in an online Ph.D. thesis by R. Boyanapalli at www.ohiolink.edu/etd/.

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