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Strains of the Harmful Cyanobacterium *Microcystis aeruginosa* Differ in Gene Expression and Activity of Inorganic Carbon Uptake Systems at Elevated CO₂ Levels

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Cyanobacteria are generally assumed to be effective competitors at low CO₂ levels because of their efficient CO₂-concentrating mechanism (CCM), and yet how bloom-forming cyanobacteria respond to rising CO₂ concentrations is less clear. Here, we investigate changes in CCM gene expression at ambient CO₂ (400 ppm) and elevated CO₂ (1,100 ppm) in six strains of the harmful cyanobacterium *Microcystis*. All strains downregulated *cmpA* encoding the high-affinity bicarbonate uptake system BCT1, whereas both the low- and high-affinity CO₂ uptake genes were expressed constitutively. Four strains downregulated the bicarbonate uptake genes *bicA* and/or *sbtA*, whereas two strains showed constitutive expression of the *bicA-sbtA* operon. In one of the latter strains, a transposon insert in *bicA* caused low *bicA* and *sbtA* transcript levels, which made this strain solely dependent on BCT1 for bicarbonate uptake. Activity measurements of the inorganic carbon (C_i) uptake systems confirmed the CCM gene expression results. Interestingly, genes encoding the RuBisCO enzyme, structural carboxysome components, and carbonic anhydrases were not regulated. Hence, *Microcystis* mainly regulates the initial uptake of inorganic carbon, which might be an effective strategy for a species experiencing strongly fluctuating C_i concentrations. Our results show that CCM gene regulation of *Microcystis* varies among strains. The observed genetic and phenotypic variation in CCM responses may offer an important template for natural selection, leading to major changes in the genetic composition of harmful cyanobacterial blooms at elevated CO₂.

CO₂ concentrations in the atmosphere may double during this century (1). In marine ecosystems, enhanced dissolution of atmospheric CO₂ causes ocean acidification (2, 3). In freshwaters, however, CO₂ concentrations may vary widely. In many lakes, the dissolved CO₂ concentration exceeds the concentration expected from equilibrium with the atmosphere due to the input of large amounts of organic carbon from terrestrial systems (4, 5). Conversely, the photosynthetic activity of dense phytoplankton blooms can deplete the CO₂ concentration far below atmospheric levels, which increases pH and makes bicarbonate the most abundant inorganic carbon species (6–8). Cyanobacteria are often considered to be very successful competitors at low CO₂ levels (9, 10), and global warming is predicted to favor an expansion of cyanobacterial blooms in eutrophic waters (11–13). However, the response of bloom-forming cyanobacteria to elevated CO₂ levels is not yet well understood.

Cyanobacteria typically use a CO₂-concentrating mechanism (CCM) with up to five different uptake systems for inorganic carbon (C_i): three for bicarbonate and two for CO₂ uptake (14). The two sodium-dependent bicarbonate uptake systems, BicA and SbtA, are present in some but not all freshwater cyanobacteria (15–17). BicA combines a low affinity for bicarbonate with a high flux rate, whereas SbtA usually has a high affinity for bicarbonate but a low flux rate (15, 18). Hence, BicA operates more effectively at high bicarbonate concentrations and vice versa SbtA at low bicarbonate concentrations (15, 17). The third bicarbonate system, BCT1, is present in most freshwater cyanobacteria and resembles SbtA with a high affinity for bicarbonate and a low flux rate. However, in contrast to SbtA, BCT1 does not require sodium and is directly ATP dependent (19). The two CO₂ uptake systems, NDH-I₃ and NDH-I₄, are also present in most freshwater cyanobacteria and convert the passively diffusing CO₂ inside the cell into bicarbonate via NADPH-driven electron flow (20). NDH-I₃

combines a high affinity for CO₂ with a low flux rate, whereas NDH-I₄ combines a low affinity with a high flux rate (20, 21). Inside the cyanobacterial cells, bicarbonate accumulates in the cytoplasm and then diffuses into the carboxysomes, where it is converted back to CO₂ via carbonic anhydrases to enable efficient carbon fixation by RuBisCO (14).

Microcystis is a harmful cyanobacterium that forms dense blooms in lakes all over the world (22–24). Moreover, many strains are capable of producing microcystin, which is a powerful hepatotoxin for birds, mammals, and humans (25, 26). Recently, it was shown that *Microcystis* strains show considerable genetic diversity in their C_i uptake system genes (17). Some strains lack the *sbtA* gene (genotype I), other strains lack *bicA* (genotype II), and again others contain the genes for all five C_i uptake systems (genotype III). Some strains also acquired transposon inserts in the *bicA-sbtA* operon (17), but it is unknown whether this affects the expression of *bicA* and *sbtA* in these strains. Although the expression of the CCM genes has been extensively studied in the model cyanobacterium *Synechocystis* sp. strain

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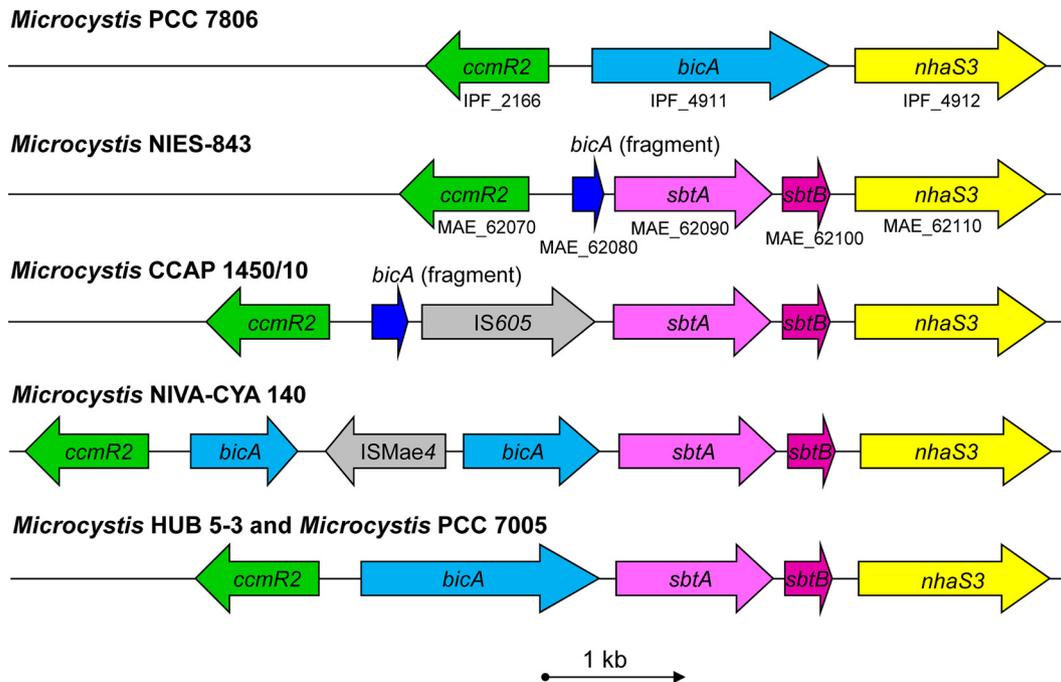


FIG 1 The *bicA-sbtA* operon of the six *Microcystis* strains of the present study. Gene *ccmR2* encodes a LysR transcriptional regulator that most likely regulates the expression of the *bicA-sbtA* operon. Some strains contain a complete *bicA* gene, whereas others only contain a small fragment. The gray arrows (IS605 and ISMae4) indicate transposon inserts. Gene *sbtB* encodes a protein that regulates the activity of SbtA (18). Gene *nhaS3* encodes a proton/sodium antiporter. The IPF numbers are the locus tags of PCC 7806, and the MAE numbers are the locus tags of NIES-843. The protein sequence identity between the *Microcystis* strains is 99% for BicA and 97 to 99% for SbtA.

PCC 6803 (27–30), CCM gene expression patterns of the environmentally relevant cyanobacterium *Microcystis* have attracted only recent interest (31–33).

In this study, we compare expression of the C_i uptake genes in response to changing CO₂ conditions among *Microcystis* strains representative of the different C_i uptake genotypes. Studies with *Synechocystis* PCC 6803 showed that genes for the high-affinity uptake systems for CO₂ (NDH-I₃) and bicarbonate (SbtA and BCT1) are induced at low CO₂, whereas the expression of low-affinity C_i uptake systems (BicA and NDH-I₄) remains unaltered (29, 30). It seems likely that the same expression patterns apply to *Microcystis*, although the presence of different C_i uptake genotypes could lead to variation in gene expression among *Microcystis* strains. We therefore defined two hypotheses for our study: (i) high-affinity C_i uptake genes are downregulated at elevated CO₂ (1,100 ppm), whereas (ii) low-affinity C_i uptake genes are expressed constitutively in *Microcystis*. To investigate these hypotheses, we compared the CCM gene expression of six *Microcystis* strains at ambient and elevated CO₂ levels. Furthermore, we measured O₂ evolution of the strains exposed to different C_i conditions to compare the activity of their C_i uptake systems. The results reveal an unexpected diversity of CO₂ responses within the genus *Microcystis*.

MATERIALS AND METHODS

Microcystis strains. We studied six *Microcystis* strains with different C_i uptake systems (Fig. 1). The C_i uptake genotypes of these strains were described in a previous study (17). Strain PCC 7806 contains the *bicA* gene but lacks the *sbtA* gene and belongs to C_i uptake genotype I. Strains NIES-843 and CCAP 1450/11 contain the *sbtA* gene but lack a complete *bicA* gene and hence belong to genotype II. Strain NIVA-CYA 140 combines *sbtA* with a complete *bicA* gene that is no longer functional because of a

transposon insert, and therefore this strain was also assigned to genotype II. Strains HUB 5-3 and PCC 7005 contain both the *bicA* and *sbtA* genes in the same operon and are therefore assigned to genotype III (Fig. 1). The four genes encoding the high-affinity bicarbonate transporter BCT1 (*cm-pABCD*), as well as the genes encoding the high-affinity CO₂ uptake system NDH-I₃ (*chpY*, *ndhD3*, *ndhF3*, and other *ndh* genes) and the low-affinity CO₂ uptake system NDH-I₄ (*chpX*, *ndhD4*, *ndhF4*, and other *ndh* genes), were present in all six strains (17). Moreover, genome-wide microarray analysis showed that *Microcystis* PCC 7806 expresses all its CCM genes (32).

Experimental setup. We used the exponential phase of batch culture experiments exposed to ambient pCO₂ (400 ppm) and elevated pCO₂ (1100 ppm) in the gas flow to study gene expression and activity of the C_i uptake systems.

First, the six *Microcystis* strains were precultured in 1-liter Erlenmeyer flasks in modified BG11 medium (34; with 10 mmol liter⁻¹ NaNO₃ and no added Na₂CO₃/NaHCO₃) for 1 week. The precultures were incubated at 25°C with 400 ppm of pCO₂ and at 120 rpm in an orbital shaker incubator (Gallenkamp, Leicester, United Kingdom), with light provided by TL-D 30W/33-640 white fluorescent tubes (Philips, Eindhoven, The Netherlands) at 20 μmol photons m⁻² s⁻¹. Microscopy checks did not reveal any contaminations.

Subsequently, new 1-liter Erlenmeyer flasks with 400 ml of modified BG11 medium were inoculated with the exponentially growing precultures at an optical density at 750 nm (OD₇₅₀) of ~0.080. Four biological replicates were used for each strain. The Erlenmeyer flasks were topped with foam stoppers to allow gas exchange and placed in an Infors HT Multitron Pro incubator (Infors Benelux, Doetinchem, The Netherlands) at 25°C with 400 ppm of pCO₂ and shaken at 120 rpm. Light was provided by white fluorescent tubes (Gro-lux F36W/Gro-T8; Havells-Sylvania Germany GmbH, Erlangen, Germany). It was recently shown that microcystins can bind to RuBisCO during oxidative stress (35), which may affect CO₂ fixation. Therefore, we decided to use low light levels of 20

$\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ during the batch culture experiments to minimize possible effects of microcystins on carbon fixation. After 2 days at 400 ppm, the pCO_2 in the incubator was increased to 1,100 ppm. The pCO_2 concentration in the gas mixture was checked regularly with an environmental gas monitor for CO_2 (EGM-4; PP Systems, Amesbury, MA). The flasks were sampled on a daily basis, and after 4 days the experiment was ended.

pH, dissolved inorganic carbon (DIC), and cell counts. The pH was measured immediately after sampling with a Lab 860 pH meter in combination with a BlueLine 28 Gel pH electrode (Schott Instruments GmbH, Mainz, Germany). To determine the concentrations of DIC, culture samples were immediately pelleted (5 min at $4,000 \times g$ and 20°C). Supernatant was filtered over $0.45\text{-}\mu\text{m}$ -pore-size 47-mm polyethersulfone membrane filters (Sartorius AG, Goettingen, Germany). The filtrate was transferred to sterile plastic urine analysis tubes (VF-109SURI; Terumo Europe N.V., Leuven, Belgium), which were filled completely (using a needle to leave all air out), and stored at 4°C until analysis. A TOC- V_{CPH} TOC analyzer (Shimadzu, Kyoto, Japan) was used to determine the DIC (measured 3- to 5-fold per sample). DIC concentrations were converted to $\text{CO}_2(\text{aq})$, bicarbonate, and carbonate concentrations using the measured pH of the samples (36). Cell numbers and biovolumes of samples from the different cultures were determined in triplicate using a Casy 1 TTC cell counter with a $60\text{-}\mu\text{m}$ capillary (Schärfe System GmbH, Reutlingen, Germany). Because the strains differed in cell size, we report the cyanobacterial abundances as biovolumes.

RNA extraction. Just before and 20 h after increasing pCO_2 from 400 to 1,100 ppm, 40-ml samples were taken for reverse transcription-quantitative PCR (RT-qPCR) analysis, immediately cooled on ice, and centrifuged for 5 min at $4,000 \times g$ and 4°C in a precooled centrifuge. The pellets were immediately resuspended in 1 ml of TRIzol (Life Technologies, Grand Island, NY), frozen in liquid nitrogen, and stored at -80°C . Subsequent RNA extraction and purification was performed as described previously (32). RNA concentrations were quantified by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, San Jose, CA), and all RNA samples had A_{260}/A_{280} and A_{260}/A_{230} values above 1.8.

RT-qPCR analysis. We investigated the expression of CCM genes using primers designed in this and previous studies (17, 32) (see also Table S1 in the supplemental material). The transcripts of the following CCM genes were targeted: *cmpA* (encoding a subunit of the high-affinity bicarbonate transporter BCT1), *bicA* and *sbtA* (the two sodium-dependent bicarbonate transporters), *chpX* (dehydration subunit of the low-affinity CO_2 uptake system NDH- I_4), *chpY* (dehydration subunit of the high-affinity CO_2 uptake system NDH- I_3), *ccmR* and *ccmR2* (two CCM transcriptional regulators), *rbcX* (chaperone for RuBisCO), *ccmM* (structural component of the carboxysomes), *ccaA1* and *ccaA2* (two carboxysomal carbonic anhydrases), and *ecaA* (periplasmic carbonic anhydrase). In addition, we targeted transcripts of the 16S rRNA gene and the *mcyB* gene (microcystin synthetase).

Reverse transcription reactions were done as described previously (32), using Superscript III (Life Technologies, Grand Island, NY). Subsequently, the qPCR Maxima SYBR green master mix (2 \times ; Thermo Fisher Scientific, Pittsburgh, PA) was applied with our primers to the obtained cDNA samples as described previously (32) to analyze PCR amplification in a ABI 7500 real-time PCR device (Applied Biosystems, Foster City, CA). The two-step cycling protocol was used, with a denaturation temperature of 95°C (15 s) and a combined annealing/extension temperature of 60°C (60 s) during 40 cycles. Melting-curve analysis was performed on all measured samples to rule out nonspecific PCR products. ROX solution (passive reference dye) was used to correct for any well-to-well variation.

We calculated relative changes in gene expression after 20 h of elevated pCO_2 using the 16S rRNA gene as a reference gene. The LinRegPCR software tool version 2012.3 (37, 38) was used for baseline correction, calculation of quantification cycle (C_q) values and calculation of the amplification efficiency (E) of each individual run using linear regression (see Table S1 in the supplemental material). Amplification efficiencies of in-

dividual samples were between 1.8 and 2.0. The relative changes in gene expression were calculated with the comparative cycle threshold (C_{T7}) method (39).

One-tailed t tests were applied to identify significant changes in gene expression ($n = 4$ biological replicates), using the false discovery rate (FDR) to correct for multihypothesis testing (55). FDR-adjusted P values of <0.05 combined with \log_2 expression changes of <-0.8 or >0.8 were considered significant.

O_2 evolution experiments. We studied the activity of different C_i uptake systems of *Microcystis* strains acclimated to low or high CO_2 levels using O_2 measurements with an Oxy-4 mini O_2 optode (PreSens GmbH, Regensburg, Germany). In mineral medium without nitrate, the initial O_2 evolution rate reflects the C_i uptake rate of cyanobacteria (32, 40). Cells acclimated to low CO_2 levels were obtained from batch cultures exposed to 400 ppm pCO_2 for 4 days. As a result, the C_i availability of the mineral medium was low, $\text{CO}_2(\text{aq})$ was depleted to $0.0018 \pm 0.0004 \mu\text{mol liter}^{-1}$ and the bicarbonate concentration was $76 \pm 10 \mu\text{mol liter}^{-1}$ (see Fig. S1 in the supplemental material). Cells acclimated to high CO_2 levels were obtained from batch cultures exposed to 400 ppm of pCO_2 for 2 days and subsequently to 1,100 ppm of pCO_2 for 2 days. The C_i availability in these cultures was high, the $\text{CO}_2(\text{aq})$ value was $4.2 \pm 1.3 \mu\text{mol liter}^{-1}$, and the bicarbonate concentration was $1,062 \pm 43 \mu\text{mol liter}^{-1}$ (see Fig. S1 in the supplemental material).

Samples from these batch cultures were pelleted ($4,000 \times g$ for 5 min at 20°C), washed once, and then resuspended in C_i -deplete and N-deplete modified BG11 medium (no added $\text{NaCO}_3/\text{NaHCO}_3$ and NaNO_3 , but with added $0.1 \text{ mmol liter}^{-1}$ NaCl and $10 \text{ mmol liter}^{-1}$ CAPSO-KOH [pH 9.8]). The medium was aerated with N_2 gas before usage. The response of the cells was studied at pH 9.8 to mimic dense blooms in which bicarbonate is the dominant C_i species. The OD_{750} of washed and resuspended samples was 0.300, and 3 ml of these samples was inserted into custom-made double-walled glass incubation chambers equipped with sensors connected to the O_2 optode device. The glass chambers were connected to a RM6 water bath (Lauda, Postfach, Germany) to keep the temperature of the samples constant at 20.3°C . Magnetic stirring was used for mixing. The O_2 optode sensors were calibrated with N_2 gas (0% oxygen) and pressurized air (21% oxygen). A saturating amount of light was provided by KL1500 compact Schott lamps (Schott AG, Mainz, Germany). Saturating light levels lead to high O_2 evolution rates, which facilitate detection of differences between the treatments. In pilot experiments, we found that the photosynthetic rates (expressed per chlorophyll a [chl a]) of strains PCC 7806 and PCC 7005 were saturated at $\sim 400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and did not decrease up to $1,000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Therefore, we used an incident light intensity (I_{in}) of $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for the O_2 evolution experiments.

At the start of the experiments, cells were allowed to take up all remaining C_i in the incubation chambers, which was monitored by a gradual decrease of the O_2 evolution. Subsequently, the rate of O_2 evolution ($\text{mg liter}^{-1} \text{ min}^{-1}$) was measured during 15-min intervals in a control treatment without additions and after adding 20, 300, or 10,000 $\mu\text{mol liter}^{-1}$ of KHCO_3 in the presence of different concentrations of NaCl and LiCl (Table 1). The different bicarbonate concentrations stimulate different C_i uptake systems. Sodium ions were added to stimulate the sodium-dependent bicarbonate transporters BicA and SbtA, whereas lithium ions were added to block bicarbonate uptake. In total, we applied six different treatments, which each activated or suppressed one or more different C_i uptake systems (Table 1). The units were converted to $\mu\text{mol O}_2 \cdot \text{mg}^{-1} \text{ chl } a \cdot \text{min}^{-1}$ using data from chlorophyll a measurements that were acquired with HPLC as described previously (32). For each strain acclimated at low or high pCO_2 levels, we tested whether O_2 evolution rates were different between the treatments using one-way analysis of variance with post hoc comparison of the means based on Tukey's HSD test ($\alpha = 0.05$; $n = 4$ per treatment).

TABLE 1 Treatments in the O₂ evolution experiments to study activity of the different C_i uptake systems^a

Treatment			Dissolved inorganic carbon		Active C _i uptake system(s)
KHCO ₃ (μmol liter ⁻¹)	NaCl (mmol liter ⁻¹)	LiCl (mmol liter ⁻¹)	CO ₂ (aq) (μmol liter ⁻¹)	HCO ₃ ⁻ (μmol liter ⁻¹)	
0	0.1	0	0	0	None (control)
20	0.1	0	0.006	15.80	BCT1
20	25	0	0.006	15.80	BCT1, SbtA
300	25	0	0.090	236.7	BCT1, SbtA, BicA
300	25	25	0.090	236.7	None
10,000	25	25	3.000	7891	NDH-I ₃ , NDH-I ₄

^a Different concentrations of KHCO₃, NaCl, and LiCl were added to induce or block the activity of specific C_i uptake systems. The resulting CO₂(aq) and HCO₃⁻ concentrations expected at pH 9.8 and 20.3°C are shown. The last column indicates which C_i uptake systems are mostly active at the applied conditions.

RESULTS

Changes in DIC and pH at elevated CO₂. Six *Microcystis* strains (PCC 7806, NIES-843, CCAP 1450/10, NIVA-CYA 140, HUB 5-3, and PCC 7005) with different C_i uptake systems were grown under atmospheric pCO₂ conditions of 400 ppm (Fig. 2). Assuming equilibrium with this atmospheric pressure, one would expect a CO₂(aq) concentration of 13.5 μmol liter⁻¹, and the pH of the mineral medium without cells would be ~7. However, owing to the photosynthetic activity of the *Microcystis* population, the CO₂(aq) concentration was depleted to <0.1 μmol liter⁻¹, the

bicarbonate concentration was <330 μmol liter⁻¹, and the pH increased to 10 to 11 depending on the strain (Fig. 2 and 3). After 2 days, we raised the pCO₂ in the gas flow to 1,100 ppm. As a consequence, the CO₂(aq) concentration increased to 1 to 12 μmol liter⁻¹ depending on the strain, the bicarbonate concentration increased to 800 to 1,500 μmol liter⁻¹, and the pH dropped 1 to 2 U (Fig. 2 and 3).

Changes in gene expression at elevated CO₂. The expression of selected CCM genes was monitored before and 20 h after increasing the pCO₂ in the gas flow (Fig. 4 and Table S2 in the

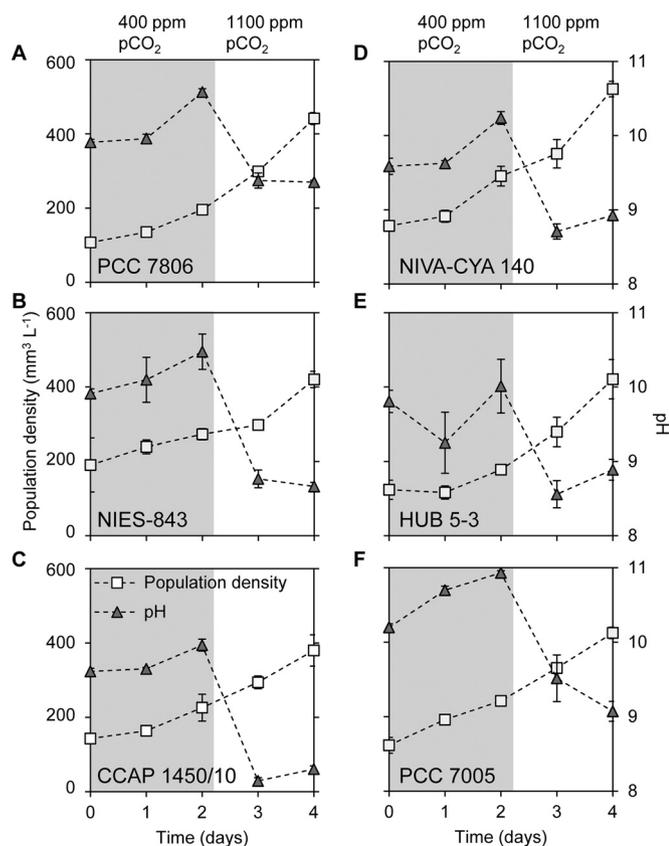


FIG 2 Growth and pH during the exponential phase in batch cultures of six *Microcystis* strains. (A) PCC 7806; (B) NIES-843; (C) CCAP 1450/10; (D) NIVA-CYA 140; (E) HUB 5-3; (F) PCC 7005. The batch cultures were exposed to 400 ppm of pCO₂ in the gas flow for 2 days (shaded area), and thereafter the pCO₂ concentration was increased to 1,100 ppm (unshaded area). The mineral medium contained 10 mmol liter⁻¹ sodium ions. Error bars indicate the standard deviations ($n = 4$ biological replicates).

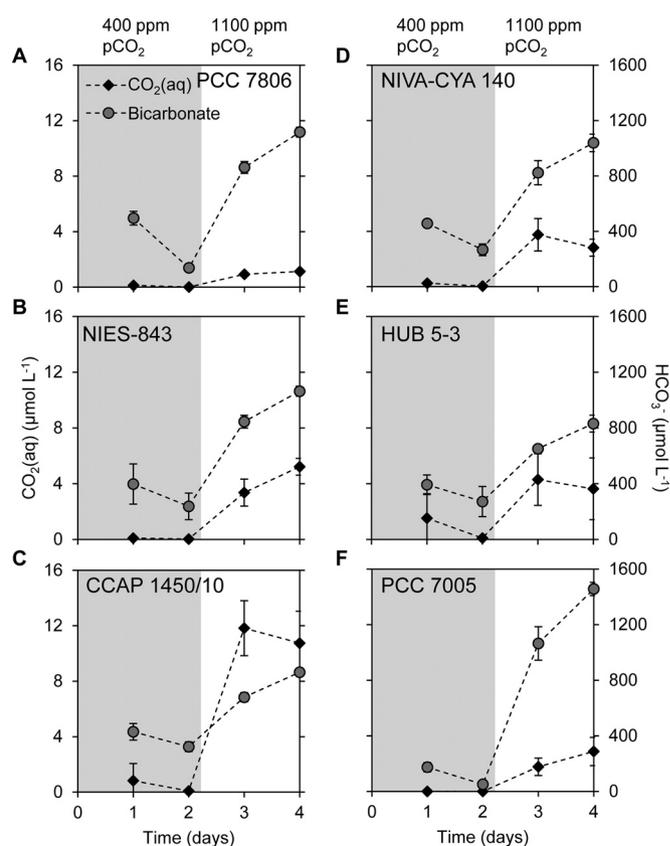


FIG 3 Dissolved CO₂ and bicarbonate concentration during the exponential phase in batch cultures of six *Microcystis* strains. (A) PCC 7806; (B) NIES-843; (C) CCAP 1450/10; (D) NIVA-CYA 140; (E) HUB 5-3; (F) PCC 7005. The batch cultures were exposed to 400 ppm of pCO₂ in the gas flow for 2 days (shaded area), and thereafter the pCO₂ concentration was increased to 1,100 ppm (unshaded area). The mineral medium contained 10 mmol liter⁻¹ sodium ions. Error bars indicate the standard deviations ($n = 4$ biological replicates).

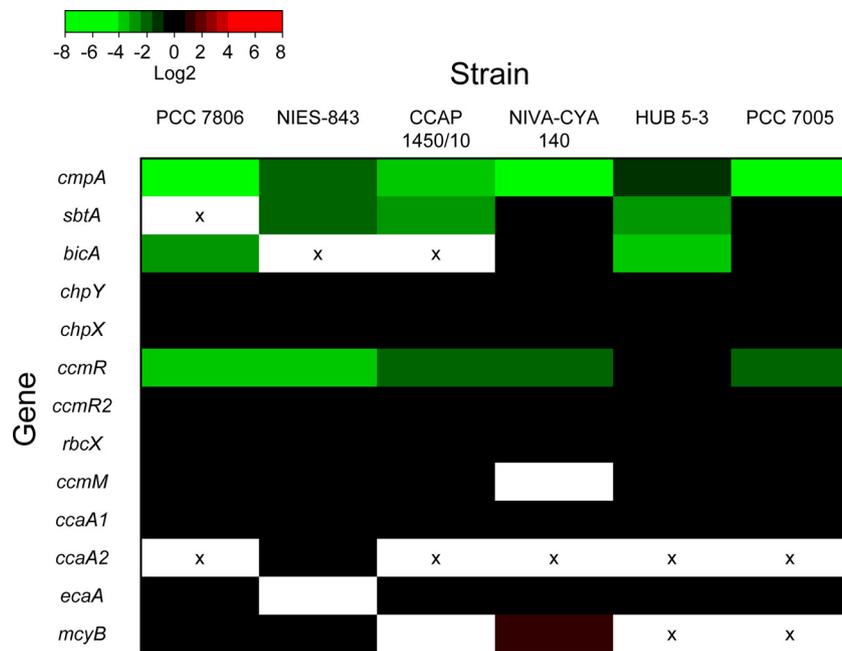


FIG 4 Heat map of changes in gene expression at elevated CO_2 for each of the six *Microcystis* strains. Gene expression changes were obtained by RT-qPCR applied to samples taken before and 20 h after increasing the pCO_2 level from 400 to 1,100 ppm. The color bar indicates \log_2 values. Significant downregulated genes are shown in green, significant upregulated genes are shown in red, and nonsignificant changes ($P > 0.05$) or \log_2 values between -0.8 and 0.8 are shown in black. Genes not measured are shown in white and genes absent in strains are marked with an “x”. Detailed RT-qPCR results are presented in Table S2 in the supplemental material.

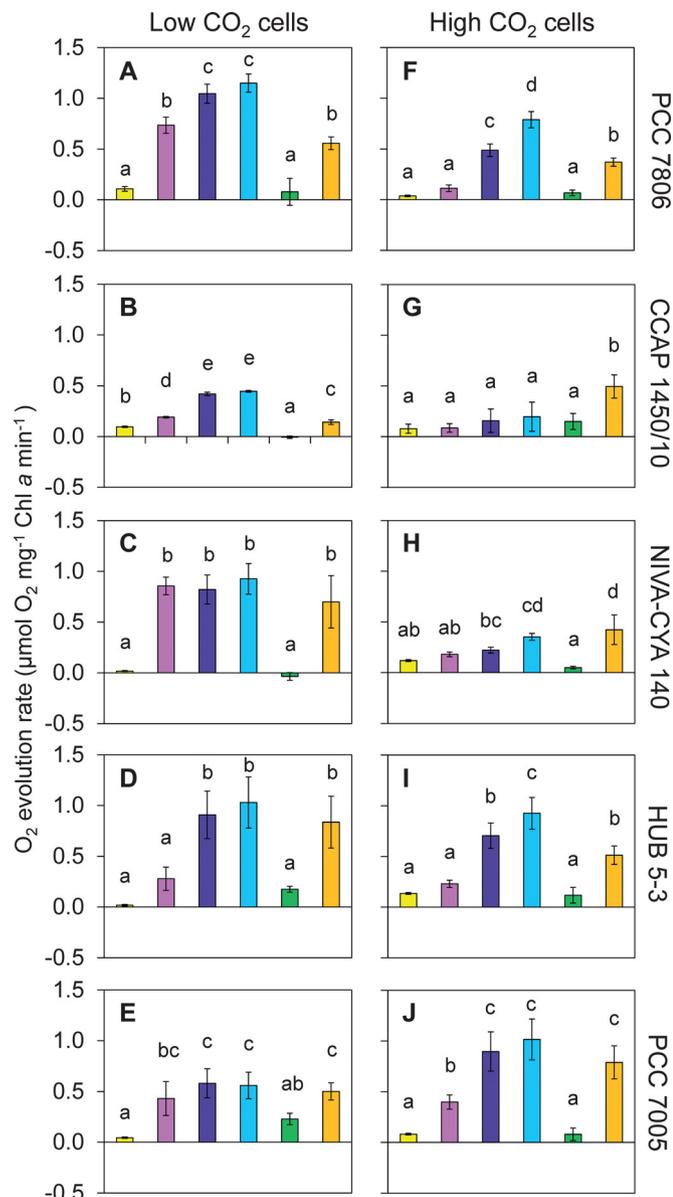
supplemental material). At an elevated pCO_2 , all strains showed significant downregulation of *cmpA*, although four strains (PCC 7806, CCAP 1450/10, NIVA-CYA 140, and PCC 7005) showed a stronger downregulation than two others (NIES-843 and HUB 5-3). The *sbtA* gene encoding the high-affinity bicarbonate transporter SbtA was significantly downregulated in strains NIES-843, CCAP 1450/10, and HUB 5-3 but was constitutively expressed in strains NIVA-CYA 140 and PCC 7005. The *bicA* gene encoding the low-affinity bicarbonate transporter BicA was downregulated in strains PCC 7806 and HUB 5-3 but was also constitutively expressed in strains NIVA-CYA 140 and PCC 7005. None of the six strains showed significant changes in gene expression of the CO_2 uptake genes *chpX* and *chpY* (Fig. 4).

Expression of the CCM transcriptional regulator *ccmR* was reduced significantly at elevated CO_2 levels in all strains, except for strain HUB 5-3. In contrast, expression of the additional transcriptional regulator *ccmR2* located upstream of the *bicA-sbtA* operon of *Microcystis* did not change significantly in any of the strains. The expression of several other CCM genes (*rbcX*, *ccmM*, *ccaA1*, *ccaA2*, and *ecaA*) was also not affected by elevated CO_2 in any of the strains. The expression of *mcyB* was unaltered in PCC 7806 and NIES-843 and increased slightly but significantly in NIVA-CYA 140.

C_i uptake activity of low- and high- CO_2 acclimated cells. We studied O_2 evolution of the *Microcystis* strains to compare the activity of their C_i uptake systems when the strains were acclimated to low- or high- CO_2 conditions (Fig. 5). We applied six different treatments to activate different C_i uptake systems, as explained in Table 1. For the interpretation of these results, we note that the response of the O_2 evolution rates to the treatments can be compared within each strain at a given pCO_2 level (i.e., within the

panels of Fig. 5) but cannot be compared quantitatively among the strains or among the two different pCO_2 levels (i.e., among the panels of Fig. 5). The reason is that other factors also influence the O_2 evolution rates, for example, the pigment concentrations and amounts of PSI and PSII may differ between strains and can also change with elevated CO_2 (32). The results can be compared among strains in a relative sense, for example, two different strains can show a significant increase in O_2 evolution after addition of $20 \mu\text{mol liter}^{-1} \text{KHCO}_3$, while a third strain does not. The O_2 evolution rates of strain NIES-843 were highly variable among the biological replicates and declined strongly after 2 h. Repetition of the O_2 evolution experiments with this strain did not improve the results, indicating that NIES-843 could not withstand the incubation conditions. Therefore, we only report the results for the other five *Microcystis* strains.

For cells acclimated to low- C_i conditions, the addition of $20 \mu\text{mol liter}^{-1} \text{KHCO}_3$ in the presence of only $0.1 \text{ mmol liter}^{-1} \text{NaCl}$ induced significantly more O_2 production than the control for all strains, except for strain HUB 5-3 which showed a nonsignificant response (Fig. 5A to E). This result indicates that BCT1 was active in all strains, although its activity was low in strain HUB 5-3. Application of $20 \mu\text{mol liter}^{-1} \text{KHCO}_3$ and $25 \text{ mmol liter}^{-1} \text{NaCl}$ led to a significantly higher O_2 production than at $20 \mu\text{mol liter}^{-1} \text{KHCO}_3$ and $0.1 \text{ mmol liter}^{-1} \text{NaCl}$ for strains PCC 7806, CCAP 1450/10, and HUB 5-3. This result indicates that in response to the added sodium ions, these three strains activated their sodium-dependent bicarbonate transporter SbtA. However, strain PCC 7806 does not have SbtA, and its response might indicate activation of the other sodium-dependent bicarbonate transporter BicA. The O_2 production at $300 \mu\text{mol liter}^{-1} \text{KHCO}_3$ and $25 \text{ mmol liter}^{-1} \text{NaCl}$ was not significantly higher than at $20 \mu\text{mol}$



Activities at pH 9.8:
 ■ control (0.1 mmol L⁻¹ NaCl)
 ■ 20 µmol L⁻¹ KHCO₃; 0.1 mmol L⁻¹ NaCl
 ■ 20 µmol L⁻¹ KHCO₃; 25 mmol L⁻¹ NaCl
 ■ 300 µmol L⁻¹ KHCO₃; 25 mmol L⁻¹ NaCl
 ■ 300 µmol L⁻¹ KHCO₃; 25 mmol L⁻¹ NaCl; 25 mmol L⁻¹ LiCl
 ■ 10,000 µmol L⁻¹ KHCO₃; 25 mmol L⁻¹ NaCl; 25 mmol L⁻¹ LiCl

FIG 5 Activity of C_i uptake systems of five *Microcystis* strains, inferred from O₂ evolution. The strains were acclimated to either low CO₂ levels (A to E) or high CO₂ levels (F to J). O₂ evolution was measured after addition of different concentrations of KHCO₃, NaCl, and LiCl to induce or block the activity of specific C_i uptake systems, as indicated in Table 1. Error bars indicate standard deviations ($n = 4$ biological replicates per treatment). Different letters above the bars indicate significant differences between the treatments, as tested by one-way analysis of variance with post hoc comparison of the means based on Tukey's HSD test ($\alpha = 0.05$).

liter⁻¹ KHCO₃ and 25 mmol liter⁻¹ NaCl for any of the strains. This result indicates that cells acclimated to low-C_i conditions relied largely on the high-affinity transporters BCT1 and SbtA for their bicarbonate uptake. Subsequent addition of 25 mmol liter⁻¹ LiCl (in the presence of 300 µmol liter⁻¹ KHCO₃ and 25 mmol liter⁻¹ NaCl) blocked bicarbonate uptake and, as a consequence, the O₂ production of all strains was reduced to levels similar to that for the control treatment. Finally, the addition of 10,000 µmol liter⁻¹ KHCO₃ in the presence of 25 mmol liter⁻¹ LiCl and 25 mmol liter⁻¹ NaCl strongly increased the CO₂(aq) concentration in the medium (see Table S1 in the supplemental material) and restored O₂ production in all strains (Fig. 5A to E). Since lithium still blocked bicarbonate uptake, this result indicates that CO₂ uptake was active in all strains.

For cells acclimated to high-C_i conditions, the addition of 20 µmol liter⁻¹ KHCO₃ in the presence of only 0.1 mmol liter⁻¹ NaCl induced significantly more O₂ production than the control only for strain PCC 7005 (Fig. 5F to J). This result indicates that the high-affinity bicarbonate transporter BCT1 was hardly active in any of the strains acclimated to high-C_i conditions, except for strain PCC 7005. Application of 20 µmol liter⁻¹ KHCO₃ and 25 mmol liter⁻¹ NaCl led to a significantly higher O₂ production than at 0.1 mmol liter⁻¹ NaCl for strains PCC 7806, HUB 5-3, and PCC 7005, indicating that these strains activated their sodium-dependent bicarbonate transporter SbtA. Strain PCC 7806 does not have SbtA, and its response might indicate activation of the other sodium-dependent bicarbonate transporter BicA. The O₂ production at 300 µmol liter⁻¹ KHCO₃ and 25 mmol liter⁻¹ NaCl was significantly higher than at 20 µmol liter⁻¹ KHCO₃ and 25 mmol liter⁻¹ NaCl for strains PCC 7806 and HUB 5-3, indicating that their low-affinity but high-flux bicarbonate transporter BicA was active when cells were acclimated to high-C_i conditions. Subsequent addition of 25 mmol liter⁻¹ LiCl blocked bicarbonate uptake and, as a consequence, the O₂ production of all strains was reduced to levels similar to that for the control treatment. Finally, addition of 10,000 µmol liter⁻¹ KHCO₃ in the presence of 25 mmol liter⁻¹ LiCl and 25 mmol liter⁻¹ NaCl increased O₂ production in comparison to the control (Fig. 5F to J), indicating that CO₂ uptake was active in all strains.

DISCUSSION

Evaluation of hypotheses. Our results enable evaluation of the hypotheses that (i) high-affinity C_i uptake genes of *Microcystis* are downregulated at elevated CO₂ (1,100 ppm), whereas (ii) low-affinity but high-flux C_i-uptake genes are constitutively expressed. Consistent with the first hypothesis, our results show that the *cmpA* gene encoding the bicarbonate-binding subunit of the high-affinity bicarbonate transporter BCT1 was downregulated in all strains. Downregulation of BCT1 at elevated CO₂ could potentially be cost-effective for the cells, because bicarbonate uptake by BCT1 is expected to require 1 ATP molecule per molecule of bicarbonate (Table 2). However, the other high-affinity bicarbonate uptake gene, *sbtA*, was downregulated at elevated CO₂ in only three of the five *sbtA*-containing strains. Furthermore, the gene *chpY* encoding the dehydration subunit of the high-affinity CO₂ uptake system NDH-I₃ was not downregulated at elevated CO₂ in any of the strains. Hence, the first hypothesis applies to *cmpA* (BCT1) and partly to *sbtA* of the two high-affinity bicarbonate uptake systems but does not apply to *chpY* of the high-affinity CO₂ uptake system.

TABLE 2 Gene expression of the five different C_i uptake systems in *Microcystis* and three model cyanobacterial strains^a

C _i uptake system (genes involved)	Energy cost (21, 51, 52)	Gene expression ^b			
		<i>Microcystis</i> strains (this study)	<i>Synechocystis</i> PCC 6803 (29, 30)	<i>Synechococcus</i> PCC 7002 (50)	<i>Synechococcus</i> PCC 7942 (45, 46)
BCT1 (<i>cmpABCD</i>)	1 ATP per HCO ₃ ⁻	Inducible under low pCO ₂	Inducible under low pCO ₂	–	Inducible under low pCO ₂
SbtA (<i>sbtA</i>)	0.5 ATP per HCO ₃ ⁻	Constitutively expressed/inducible under low pCO ₂ /–	Inducible under low pCO ₂	Inducible under low pCO ₂	Inducible under low pCO ₂
BicA (<i>bicA</i>)	0.25 ATP per HCO ₃ ⁻	Constitutively expressed/inducible under low pCO ₂ /–	Constitutively expressed	Inducible under low pCO ₂	–
NDH-I ₃ (<i>chpY</i> and others)	1 NADPH per CO ₂ -to-HCO ₃ ⁻ conversion	Constitutively expressed	Inducible under low pCO ₂	Inducible under low pCO ₂	Inducible under low pCO ₂
NDH-I ₄ (<i>chpX</i> and others)	1 NADPH per CO ₂ -to-HCO ₃ ⁻ conversion	Constitutively expressed	Constitutively expressed	Constitutively expressed	Constitutively expressed

^a The estimated energy costs of the different C_i uptake systems are indicated in terms of molecules of ATP or NADPH per molecule CO₂ or HCO₃⁻. The source or reference(s) are indicated in parentheses in the headings for each column of data. A dash (–) indicates that the C_i uptake system is absent.

^b The *Microcystis* strains, *Synechocystis* PCC 6803, and *Synechococcus* PCC 7942 were from freshwater (brackish water for *Microcystis* PCC 7806); *Synechococcus* PCC 7002 has a marine origin.

Consistent with the second hypothesis, the gene *chpX* encoding the dehydration subunit of the low-affinity CO₂ uptake system NDH-I₄ was constitutively expressed in all strains. However, the low-affinity bicarbonate uptake gene, *bicA*, was constitutively expressed only in strains NIVA-CYA 140 (where it is not functional because of a transposon insert; Fig. 1) and PCC 7005 but was downregulated at elevated CO₂ in strains PCC 7806 and HUB 5-3. Hence, the second hypothesis is supported by *chpX* (NDH-I₄), whereas the low-affinity bicarbonate uptake gene *bicA* shows a more variable response.

General observations. Given that both hypotheses received only partial support, what general observations can still be obtained from the gene expression patterns of the *Microcystis* strains? First, it is noteworthy that several C_i uptake systems and one of their transcriptional regulators were regulated in response to elevated CO₂, whereas other important CCM genes encoding the enzyme RuBisCO, structural components of the carboxysome, and carbonic anhydrases were not regulated at all (Fig. 4). Furthermore, a recent transcriptome study of *Microcystis* PCC 7806 found that expression of the *ppc* gene, encoding phosphoenolpyruvate carboxylase involved in an alternative C_i assimilation pathway, also remained constant under elevated-CO₂ conditions (see Table S4 in the supplemental material in reference 32). These results indicate that the CCM genes of *Microcystis* respond to elevated CO₂ mainly at the very first steps of the carbon fixation process by regulating the initial acquisition of inorganic carbon. *Microcystis* is a buoyant cyanobacterium that can develop dense blooms in eutrophic lakes, where it will be exposed to large fluctuations in CO₂ availability at both daily and seasonal time scales (41). A highly specific response that mainly adjusts the initial C_i uptake systems, without large changes in expression of the carboxysome genes and genes of the downstream carbon assimilation pathways, could preserve energy and offer a robust strategy for a species that often experiences strongly fluctuating C_i conditions (32).

Second, all of the C_i uptake genes investigated in the present study were either downregulated or remained unchanged at elevated CO₂; none of them were upregulated. Hence, all C_i uptake

systems that a strain was capable to produce were available for the cells at low CO₂ levels, including the low-affinity C_i uptake systems. Third, the genes *chpX* and *chpY* of both CO₂ uptake systems were expressed constitutively, which might again be an adaptation to fluctuating CO₂ conditions. Constitutive expression of the high-affinity CO₂ uptake system NDH-I₃ might also be an adaptation to intercept low intracellular concentrations of CO₂ leaking from the carboxysomes.

Methodological aspects. Previously, the cellular response of strain PCC 7806 to elevated CO₂ was investigated in highly controlled chemostats using whole-genome microarrays (32). In the present study, we simplified the experimental setup to batch cultures and limited our analysis to a smaller set of genes using RT-qPCR, which enabled investigation of a larger number of strains. We included strain PCC 7806 in our present study to check the consistency of the results.

Strain PCC 7806 downregulated expression of *cmpA* and *bicA* at elevated CO₂ in both the previous and the present study (Fig. 4). Furthermore, both studies showed constitutive expression of the CO₂ uptake, carboxysomal and RuBisCO genes, and downregulation of the transcriptional regulator gene *ccmR*, although *ccmR2* was only downregulated in the previous study (32). Moreover, the O₂ evolution data of PCC 7806 show that BCT1 and BicA were both active under low-pCO₂ conditions (Fig. 5A), whereas BicA but not BCT1 was active under high-pCO₂ conditions (Fig. 5F), in agreement with the findings of the previous study (32). Hence, the results of both studies are in good agreement, which gives confidence in the applied methods.

In our O₂ evolution experiments, the cells were exposed to different salt treatments (Table 1). This could potentially bias the results because *Microcystis* strains differ in their salt tolerance and potassium ion sensitivity (42). In particular, strain PCC 7005 is very sensitive to elevated potassium ion concentrations and lacks several salt tolerance genes, whereas strain PCC 7806 is much more tolerant to potassium ions and can withstand 170 mmol liter⁻¹ NaCl (42, 43). We therefore tried to minimize salt stress by exposing the cells to the different treatments for only 15 min. The results show that even the salt- and potassium-sensitive strain

TABLE 3 Presence and function of the CCM transcriptional regulators in *Microcystis* and three model cyanobacterial strains^a

Transcriptional regulator	Location in genome	Function			
		<i>Microcystis</i> (17; this study)	<i>Synechocystis</i> PCC 6803 (29, 53)	<i>Synechococcus</i> PCC 7002 (50)	<i>Synechococcus</i> PCC 7942 (46, 54)
CcmR	Upstream of high-affinity CO ₂ uptake operon	Repressor/activator of <i>cmpABCD</i> operon (BCT1)	Repressor of <i>sbtA</i> and high-affinity CO ₂ uptake operon (not <i>bicA</i>)	Repressor of <i>sbtA</i> and <i>bicA</i> (possibly high-affinity CO ₂ uptake operon)	–
CcmR2	Upstream of <i>bicA-sbtA</i> operon	Repressor/activator of <i>bicA-sbtA</i> operon	–	–	–
CmpR	Upstream of <i>cmpABCD</i> operon (BCT1) or separate location	–	Activator of <i>cmpABCD</i> operon	–	Activator of <i>cmpABCD</i> operon (possibly a repressor of <i>sbtA</i> and high-affinity CO ₂ uptake operon)

^a The source or reference(s) are indicated in parentheses in the headings for each column of data. A dash (–) indicates that the transcriptional regulator is absent.

PCC 7005 maintained high O₂ evolution rates in the treatment with the highest salinity (10 mmol liter⁻¹ KHCO₃, 25 mmol liter⁻¹ LiCl and 25 mmol liter⁻¹ NaCl; Fig. 5), and hence the applied salinities apparently did not hinder the activity of the cells during this short time interval.

Expression and activity of the sodium-dependent C_i uptake genes. Expression of the sodium-dependent bicarbonate uptake genes *bicA* and *sbtA* varied widely among the strains. Even similar genotypes, such as strains HUB 5-3 and PCC 7005 that both belong to genotype III (*bicA-sbtA*), showed contrasting expression patterns for their bicarbonate uptake genes (Fig. 4). These two strains have *bicA* and *sbtA* located in one operon (17), and hence cotranscription explains why the expression patterns of these two genes are coupled (Fig. 4). The O₂ evolution data indicate that HUB 5-3 mainly relies on its sodium-dependent bicarbonate uptake systems under low-C_i conditions (Fig. 5D), whereas PCC 7005 mainly relies on ATP-dependent bicarbonate uptake by BCT1 (Fig. 5E). Hence, a reduction of the cellular investments in bicarbonate uptake at elevated CO₂ would be most effective by downregulation of the *bicA-sbtA* operon for HUB 5-3 and by downregulation of BCT1 for PCC 7005, a finding in line with the observed changes in gene expression (Fig. 4).

Strain NIVA-CYA 140 contains *sbtA* but has a transposon insert in the middle of a complete *bicA* gene (Fig. 1) and was therefore assigned to C_i uptake genotype II (no *bicA*) (17). At low pCO₂ the transcription level of *sbtA* (relative to 16S rRNA) was ~45× lower in this strain than in the other strains, indicating that the transposon insert interfered with transcription of the *bicA-sbtA* operon. Indeed, the strain depended strongly on BCT1 under low-pCO₂ conditions, as evidenced from the lack of stimulation by added sodium ions in the O₂ evolution experiments (Fig. 5C), whereas the strain depended mainly on CO₂ uptake under high-pCO₂ conditions (Fig. 5H). Hence, most likely this strain does not use the sodium-dependent bicarbonate uptake systems BicA and SbtA and therefore has a phenotype that deviates from all other *Microcystis* strains.

Strain CCAP 1450/10 also has a transposon insert, located between a small *bicA* fragment and a complete *sbtA* gene (Fig. 1). However, the gene expression results (Fig. 4) and the O₂ evolution data (Fig. 5B) show that the transposon insert did not hinder expression of *sbtA* in this strain.

Comparison of CCM gene regulation of *Microcystis* with other cyanobacteria. Previously, the CCM genes of the model cyanobacteria *Synechocystis* PCC 6803, *Synechococcus* PCC 7002, and *Synechococcus* 7942 were studied in detail (15, 19, 27–30, 44–48). Comparison of the CCM genes of our *Microcystis* strains with these model cyanobacteria reveals several similarities and differences (Tables 2 and 3).

In all cyanobacteria investigated thus far, genes encoding the ATP-dependent high-affinity bicarbonate transporter BCT1 are induced under low-C_i conditions, whereas genes encoding the low-affinity CO₂ uptake system NDH-I₄ are constitutively expressed (Table 2). Hence, our two hypotheses do apply to the genes of these two uptake systems. The constitutive expression of genes encoding the high-affinity CO₂ uptake system NDH-I₃ in all *Microcystis* strains and the high-affinity bicarbonate transporter SbtA in some *Microcystis* strains deviates from the induction of these genes in the other three cyanobacteria. The presence and expression of *bicA* appears to be quite variable, not only in *Microcystis* but also in other cyanobacteria.

The CCM transcriptional regulators also differ among the cyanobacteria (Table 3). CcmR can regulate transcription of several C_i uptake genes. In *Synechocystis* PCC 6803, CcmR appeared to be a repressor of *sbtA* and the high-affinity CO₂ uptake operon but not of *bicA* (29, 47). In contrast, in *Synechococcus* PCC 7002, CcmR appeared to be a repressor of *bicA* and *sbtA* and possibly the high-affinity CO₂ uptake operon (48, 50). In *Microcystis*, CcmR probably regulates expression of the *cmpABCD* operon (encoding BCT1), since downregulation of *ccmR* at elevated pCO₂ coincided with downregulation of the *cmpA* gene (Fig. 4). *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 use another transcriptional regulator, CmpR, for the *cmpABCD* operon (27, 47), which is absent from *Microcystis*. CcmR2 is the most likely transcriptional regulator for the *bicA* and *sbtA* genes in *Microcystis*, given the location of *ccmR2* upstream of the *bicA-sbtA* operon (17).

Ecological implications. In conclusion, our results reveal an unexpected diversity in CO₂ responses of cyanobacteria. It was already known that *Microcystis* strains differ in their C_i uptake genes, which promotes variation in their CO₂ response (17). Our results show that, on top of this genotypic diversity, there is also considerable phenotypic variation because strains with the same C_i uptake genes can show contrasting expression patterns and may differ widely in the activity of their C_i uptake systems. In other

words, cyanobacterial strains differ in their adaptation to changing CO₂ conditions not only because of variation in genetic composition but also because of further variation at the transcriptional and physiological level.

It is often argued that cyanobacteria generally have a very effective CCM and are therefore particularly strong competitors at low CO₂ levels in comparison to eukaryotic phytoplankton (9). However, we know now that there is major variation in the CCM tactics among cyanobacteria and even among different strains within the same genus. Some *Microcystis* strains perform well at low CO₂, whereas other strains are much better competitors under high-CO₂ conditions (17, 49). This genetic and phenotypic variation in C_i uptake systems provides cyanobacterial communities with the potential for rapid adaptation and acclimation to changing CO₂ conditions. These differential responses also indicate that the ongoing rise in atmospheric CO₂ concentrations is likely to be more beneficial for some cyanobacterial strains than for others, which may lead to major changes in the genetic composition of harmful cyanobacterial blooms.

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