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 (Ci) 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 Ci 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.

C O₂ concentrations in the atmosphere may double during this century (1). In marine ecosystems, enhanced dissolution of atmospheric CO₂ causes ocean acidification (2, 3). In freshwater systems, 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 (Ci): 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).
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 CO\(_2\) 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 CO\(_2\) (NDH-I\(_4\)) and bicarbonate (SbtA and BCT1) are induced at low CO\(_2\), whereas the expression of low-affinity C\(_i\) uptake systems (BicA and NDH-I\(_4\)) 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 CO\(_2\), whereas 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 CO\(_2\) levels. Furthermore, we measured O\(_2\) 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 CO\(_2\) 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-pABC), as well as the genes encoding the high-affinity CO\(_2\) uptake system NDH-I\(_4\) (chpY, ndhD3, ndhF3, and other ndh genes) and the low-affinity CO\(_2\) uptake system NDH-I\(_4\) (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 pCO\(_2\) (400 ppm) and elevated pCO\(_2\) (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\(^{-1}\) NaNO\(_3\) and no added Na\(_2\)CO\(_3/\)NaHCO\(_3\)) for 1 week. The precultures were incubated at 25°C with 400 ppm of pCO\(_2\) 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 \(\mu\)mol 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 incubated at 25°C with 400 ppm of pCO\(_2\) 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 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\). 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 CO\(_2\) fixation. Therefore, we decided to use low light levels of 20

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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.
μmol photons m⁻² s⁻¹ during the batch culture experiments to minimize possible effects of microysts on carbon fixation. After 2 days at 400 ppm, the pCO₂ in the incubator was increased to 1,100 ppm. The pCO₂ concentration in the gas mixture was checked regularly with an environmental gas monitor for CO₂ (EGM-4; PP Systems, Amesbury, MA). The flakes 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 × g and 20°C). Supernatant was filtered over 0.45-μm- 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-VCPH TOC analyzer (Shimadzu, Kyoto, Japan) was used to determine the DIC (measured 3- to 5-fold per sample). DIC concentrations were converted to CO₂(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-μm 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 pCO₂ 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 × 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 spectrophotometer (Thermo Scientific, San Jose, CA), and all RNA samples had A₂₆₀/A₂₈₀ and A₂₆₀/A₃₂₈ 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: ccmA (encoding a subunit of the high-affinity bicarbonate transporter BCT1), bicA and sbtA (the two sodium-dependent bicarbonate transporters), chpX (degradation subunit of the low-affinity CO₂ uptake system NDH-I₂), chpY (degradation subunit of the high-affinity CO₂ uptake system NDH-I₁), ccmR and ccmR2 (two CCM transcriptional regulators), rhCX (chaperone for Rubisco), ccmM (structural component of the carboxyoxanes), 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 (2X; 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 pCO₂ 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 (Cq) 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 individual samples were between 1.8 and 2.0. The relative changes in gene expression were calculated with the comparative cycle threshold (Ct) 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 log₂ expression changes of −0.8 or >0.8 were considered significant.

**O₂ evolution experiments.** We studied the activity of different C₄ uptake systems of Microcystis strains acclimated to low or high CO₂ levels using O₂ measurements with an Oxy-4 mini O₂ optode (PreSens GmbH, Regensburg, Germany). In mineral medium without nitrate, the initial O₂ evolution rate reflects the C₄ uptake rate of cyanobacteria (32, 40). Cells acclimated to low CO₂ levels were obtained from batch cultures exposed to 400 ppm pCO₂ for 4 days. As a result, the C₄ availability of the mineral medium was low, CO₂(aq) was depleted to 0.0018 ± 0.0004 μmol liter⁻¹ and the bicarbonate concentration was 76 ± 10 μmol liter⁻¹ (see Fig. S1 in the supplemental material). Cells acclimated to high CO₂ levels were obtained from batch cultures exposed to 400 ppm pCO₂ for 2 days and subsequently to 1,100 ppm of pCO₂ for 2 days. The C₄ availability in these cultures was high, the CO₂(aq) value was 4.2 ± 1.3 μmol liter⁻¹, and the bicarbonate concentration was 1.062 ± 43 μmol liter⁻¹ (see Fig. S1 in the supplemental material).

Samples from these batch cultures were pelleted (4,000 × g for 5 min at 20°C), washed once, and then resuspended in C₆-deplete and N-deplete modified BG11 medium (no added NaCO₃/NaHCO₃ and NaNO₃), but with added 0.1 mmol liter⁻¹ NaCl and 10 mmol liter⁻¹ CAPSO-KOH (pH 9.8). The medium was aerated with N₂ gas before usage. The response of the cells was studied at pH 9.8 to mimic dense blooms in which bicarbonate is the dominant C₄ species. The OD₅₀₀ 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 O₂ 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 O₂ optode sensors were calibrated with N₂ 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 led to high O₂ 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]₅₅₅) of strains PCC 7806 and PCC 7005 were saturated at −400 μmol photons m⁻² s⁻¹ and did not decrease up to 1,000 μmol photons m⁻² s⁻¹. Therefore, we used an incident light intensity (I₉₅) of 500 μmol photons m⁻² s⁻¹ for the O₂ evolution experiments.

At the start of the experiments, cells were allowed to take up all remaining C₄ in the incubation chambers, which was monitored by a gradual decrease of the O₂ evolution. Subsequently, the rate of O₂ evolution (μg liter⁻¹ min⁻¹) was measured during 15-min intervals in a control treatment without additions and after adding 20, 300, or 10,000 μmol liter⁻¹ of KHCO₃ in the presence of different concentrations of NaCl and LiCl (Table 1). The different bicarbonate concentrations stimulate different C₄ 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 C₄ uptake systems (Table 1). The units were converted to μmol O₂ · mg⁻¹ chl a · 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 pCO₂ levels, we tested whether O₂ 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 (α = 0.05; n = 4 per treatment).
RESULTS

Changes in DIC and pH at elevated CO$_2$. 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 pCO$_2$ conditions of 400 ppm (Fig. 2). Assuming equilibrium with this atmospheric pressure, one would expect a CO$_2$(aq) concentration of 13.5 mol 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 CO$_2$(aq) concentration was depleted to 0.1 mol liter$^{-1}$, the bicarbonate concentration was ~330 mol liter$^{-1}$, and the pH increased to 10 to 11 depending on the strain (Fig. 2 and 3). After 2 days, we raised the pCO$_2$ in the gas flow to 1,100 ppm. As a consequence, the CO$_2$(aq) concentration increased to 1 to 12 mol liter$^{-1}$ depending on the strain, the bicarbonate concentration increased to 800 to 1,500 mol liter$^{-1}$, and the pH dropped 1 to 2 U (Fig. 2 and 3).

Changes in gene expression at elevated CO$_2$. The expression of selected CCM genes was monitored before and 20 h after increasing the pCO$_2$ in the gas flow (Fig. 4 and Table S2 in the

<table>
<thead>
<tr>
<th>Treatment Dissolved inorganic carbon</th>
<th>KHCO$_3$ (µmol liter$^{-1}$)</th>
<th>NaCl (mmol liter$^{-1}$)</th>
<th>LiCl (mmol liter$^{-1}$)</th>
<th>CO$_2$(aq) (µmol liter$^{-1}$)</th>
<th>HCO$_3^-$ (µmol liter$^{-1}$)</th>
<th>Active C$_i$ uptake system(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>None (control)</td>
</tr>
<tr>
<td>20</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0.006</td>
<td>15.80</td>
<td>BCT1</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0.006</td>
<td>15.80</td>
<td>BCT1, SbtA</td>
</tr>
<tr>
<td>300</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0.090</td>
<td>236.7</td>
<td>BCT1, SbtA, BicA</td>
</tr>
<tr>
<td>300</td>
<td>25</td>
<td>25</td>
<td>0</td>
<td>0.090</td>
<td>236.7</td>
<td>None</td>
</tr>
<tr>
<td>10,000</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>3.000</td>
<td>7891</td>
<td>NDH-I3, NDH-I4</td>
</tr>
</tbody>
</table>

*Different concentrations of KHCO$_3$, NaCl, and LiCl were added to induce or block the activity of specific C$_i$ uptake systems. The resulting CO$_2$(aq) and HCO$_3^-$ 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.*

![FIG 2](https://example.com/figure2.png) 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 pCO$_2$ in the gas flow for 2 days (shaded area), and thereafter the pCO$_2$ 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](https://example.com/figure3.png) Dissolved CO$_2$ 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 pCO$_2$ in the gas flow for 2 days (shaded area), and thereafter the pCO$_2$ 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).
supplemental material). At an elevated pCO₂, 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 CO₂ uptake genes chpX and chpY (Fig. 4).

Expression of the CCM transcriptional regulator ccmR was reduced significantly at elevated CO₂ 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 ccaA) was also not affected by elevated CO₂ 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.

**C₅ uptake activity of low- and high-CO₂ acclimated cells.** We studied O₂ evolution of the Microcystis strains to compare the activity of their C₅ uptake systems when the strains were acclimated to low- or high-CO₂ conditions (Fig. 5). We applied six different treatments to activate different C₅ uptake systems, as explained in Table 1. For the interpretation of these results, we note that the response of the O₂ evolution rates to the treatments can be compared within each strain at a given pCO₂ level (i.e., within the panels of Fig. 5) but cannot be compared quantitatively among the strains or among the two different pCO₂ levels (i.e., among the panels of Fig. 5). The reason is that other factors also influence the O₂ evolution rates, for example, the pigment concentrations and amounts of PSI and PSII may differ between strains and can also change with elevated CO₂ (32). The results can be compared among strains in a relative sense, for example, two different strains can show a significant increase in O₂ evolution after addition of 20 μmol liter⁻¹ KHCO₃ while a third strain does not. The O₂ evolution rates of strain NIES-843 were highly variable among the biological replicates and declined strongly after 2 h. Repetition of the O₂ 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-C₅ 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 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 μmol liter⁻¹ KHCO₃ and 25 mmol liter⁻¹ NaCl led to a significantly higher O₂ production than at 20 μmol liter⁻¹ KHCO₃ and 0.1 mmol liter⁻¹ 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 O₂ production at 300 μmol liter⁻¹ KHCO₃ and 25 mmol liter⁻¹ NaCl was not significantly higher than at 20 μmol...
O2 evolution. The strains were acclimated to either low CO2 levels (A to E) or specific Ci 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 Tukey’s HSD test ($\alpha = 0.05$).

One-way analysis of variance with post hoc comparison of the means based on the bars indicate significant differences between the treatments, as tested by Tukey’s HSD test ($\alpha = 0.05$).

For cells acclimated to high-Ci conditions, the addition of 20 $\mu$mol liter$^{-1}$ KHCO$_3$ in the presence of only 0.1 $\mu$mol 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-Ci conditions, except for strain PCC 7005. Application of 20 $\mu$mol liter$^{-1}$ KHCO$_3$ and 25 $\mu$mol liter$^{-1}$ NaCl led to a significantly higher O2 production than at 0.1 $\mu$mol 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 BiA. The O2 production at 300 $\mu$mol liter$^{-1}$ KHCO$_3$ and 25 $\mu$mol liter$^{-1}$ NaCl was significantly higher than at 20 $\mu$mol liter$^{-1}$ KHCO$_3$ and 25 $\mu$mol liter$^{-1}$ NaCl for strains PCC 7806 and HUB 5-3, indicating that their low-affinity but high-flux bicarbonate transporter BiA was active when cells were acclimated to high-Ci conditions. Subsequent addition of 25 $\mu$mol 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 $\mu$mol liter$^{-1}$ KHCO$_3$ in the presence of 25 $\mu$mol liter$^{-1}$ LiCl and 25 $\mu$mol 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.

**DISCUSSION**

**Evaluation of hypotheses.** Our results enable evaluation of the hypotheses that (i) high-affinity Ci uptake genes of *Microcystis* are downregulated at elevated CO2 (1,100 ppm), whereas (ii) low-affinity but high-flux Ci-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-1$_3$ 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.
Consistent with the second hypothesis, the gene *chpX* encoding the dehydration subunit of the low-affinity CO$_2$ uptake system NDH-I$_4$ 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 CO$_2$ in strains PCC 7806 and HUB 5-3. Hence, the second hypothesis is supported by *chpX* (NDH-I$_4$), 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 Ci uptake systems and one of their transcriptional regulators were regulated in response to elevated CO$_2$, 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 Ci assimilation pathway, also remained constant under elevated-CO$_2$ conditions (see Table S4 in the supplemental material in reference 32). These results indicate that the CCM genes of *Microcystis* respond to elevated CO$_2$, 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 CO$_2$ availability at both daily and seasonal time scales (41). A highly specific response that mainly adjusts the initial Ci 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 Ci conditions (32).

Second, all of the Ci uptake genes investigated in the present study were either downregulated or remained unchanged at elevated CO$_2$; none of them were upregulated. Hence, all Ci uptake systems that a strain was capable to produce were available for the cells at low CO$_2$ levels, including the low-affinity Ci uptake systems. Third, the genes *chpX* and *chpY* of both CO$_2$ uptake systems were expressed constitutively, which might again be an adaptation to fluctuating CO$_2$ conditions. Constitutive expression of the high-affinity CO$_2$ uptake system NDH-I$_4$ might also be an adaptation to intercept low intracellular concentrations of CO$_2$ leaking from the carboxysomes.

**Methodological aspects.** Previously, the cellular response of strain PCC 7806 to elevated CO$_2$ 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 CO$_2$ in both the previous and the present study (Fig. 4). Furthermore, both studies showed constitutive expression of the CO$_2$ 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 O$_2$ evolution data of PCC 7806 show that BCT1 and BicA were both active under low-pCO$_2$ conditions (Fig. 5A), whereas BicA but not BCT1 was active under high-pCO$_2$ 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 O$_2$ 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

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

<table>
<thead>
<tr>
<th>Ci uptake system (genes involved)</th>
<th>Energy cost</th>
<th>Gene expression$^b$</th>
<th><em>Synechocystis</em> PCC 6803 (29, 30)</th>
<th><em>Synechococcus</em> PCC 7002 (50)</th>
<th><em>Synechococcus</em> PCC 7942 (45, 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCT1 (<em>cmpABCD</em>)</td>
<td>1 ATP per HCO$_3^-$</td>
<td>Inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
</tr>
<tr>
<td>SbtA (<em>sbtA</em>)</td>
<td>0.5 ATP per HCO$_3^-$</td>
<td>Constitutively expressed/inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
</tr>
<tr>
<td>BicA (<em>bicA</em>)</td>
<td>0.25 ATP per HCO$_3^-$</td>
<td>Constitutively expressed/inducible under low pCO$_2$</td>
<td>Constitutively expressed</td>
<td>Inducible under low pCO$_2$</td>
<td>–</td>
</tr>
<tr>
<td>NDH-I$_4$ (<em>chpY</em> and others)</td>
<td>1 NADPH per CO$_2$-to-HCO$_3^-$ conversion</td>
<td>Constitutively expressed</td>
<td>Inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
<td>Inducible under low pCO$_2$</td>
</tr>
<tr>
<td>NDH-I$_4$ (<em>chpX</em> and others)</td>
<td>1 NADPH per CO$_2$-to-HCO$_3^-$ conversion</td>
<td>Constitutively expressed</td>
<td>Constitutively expressed</td>
<td>Constitutively expressed</td>
<td>Constitutively expressed</td>
</tr>
</tbody>
</table>

$^a$ The estimated energy costs of the different Ci uptake systems are indicated in terms of molecules of ATP or NADPH per molecule CO$_2$ or HCO$_3^-$.

$^b$ 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.
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 C₄ uptake genes.** Expression of the sodium-dependent bicarbonate uptake genes \( \text{bicA} \) and \( \text{sbtA} \) varied widely among the strains. Even similar genotypes, such as strains HUB 5-3 and PCC 7005 that express patterns for their bicarbonate uptake genes (Fig. 4).

These two strains have \( \text{bicA} \) and \( \text{sbtA} \) 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-C₄ 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 \( \text{bicA-sbtA} \) operon for HUB 5-3 and by downregulation of BCT1 for PCC 7005, a finding in line with the observed changes in expression (Fig. 4).

Strain NIVA-CYA 140 contains \( \text{sbtA} \) but has a transposon inserted in the middle of a complete \( \text{bicA} \) gene (Fig. 1) and was therefore assigned to C₄ uptake genotype II (no \( \text{bicA} \)) (17). At low pCO₂ the transcription level of \( \text{sbtA} \) (relative to 16S rRNA) was ~45X lower in this strain than in the other strains, indicating that the transposon insertion interfered with transcription of the \( \text{bicA-sbtA} \) 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 \( \text{bicA} \) fragment and a complete \( \text{sbtA} \) 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 \( \text{sbtA} \) 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-C₄ 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 \( \text{bicA} \) 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 C₄ uptake genes. In *Synechocystis* PCC 6803, CcmR appeared to be a repressor of \( \text{sbtA} \) and the high-affinity CO₂ uptake operon but not of \( \text{bicA} \) (29, 47). In contrast, in *Synechococcus* PCC 7002, CcmR appeared to be a repressor of \( \text{bicA} \) and \( \text{sbtA} \) and possibly the high-affinity CO₂ uptake operon (48, 50). In *Microcystis*, CcmR probably regulates expression of the \( \text{cmpABCD} \) operon (encoding BCT1), since downregulation of \( \text{cmrR} \) at elevated pCO₂ coincided with downregulation of the \( \text{cmpA} \) gene (Fig. 4). *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 use another transcriptional regulator, CmrR, for the \( \text{cmpABCD} \) operon (27, 47), which is absent from *Microcystis*. CcmR2 is the most likely transcriptional regulator for the \( \text{bicA} \) and \( \text{sbtA} \) genes in *Microcystis*, given the location of \( \text{cmrR2} \) upstream of the \( \text{bicA-sbtA} \) 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 C₄ 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 C₄ uptake genes can show contrasting expression patterns and may differ widely in the activity of their C₄ uptake systems. In other 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 \( \text{bicA} \) appears to be quite variable, not only in *Microcystis* but also in other cyanobacteria.

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

<table>
<thead>
<tr>
<th>Transcriptional regulator</th>
<th>Location in genome</th>
<th>Function</th>
<th>Microcystis (17; this study)</th>
<th>Synechocystis PCC 6803 (29, 53)</th>
<th>Synechococcus PCC 7002 (50)</th>
<th>Synechococcus PCC 7942 (46, 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CcmR</td>
<td>Upstream of high-affinity CO₂ uptake operon</td>
<td>Repressor/activator of ( \text{cmpABCD} ) operon (BCT1)</td>
<td>High-affinity CO₂ uptake operon</td>
<td>High-affinity CO₂ uptake operon</td>
<td>High-affinity CO₂ uptake operon</td>
<td>High-affinity CO₂ uptake operon</td>
</tr>
<tr>
<td>CcmR2</td>
<td>Upstream of ( \text{bicA-sbtA} ) operon</td>
<td>Repressor/activator of ( \text{bicA-sbtA} ) operon</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CmrR</td>
<td>Upstream of ( \text{cmpABCD} ) operon (BCT1) or separate location</td>
<td>Activator of ( \text{cmpABCD} ) operon</td>
<td>Activator of ( \text{cmpABCD} ) operon</td>
<td>Activator of ( \text{cmpABCD} ) operon</td>
<td>Activator of ( \text{cmpABCD} ) operon</td>
<td>Activator of ( \text{cmpABCD} ) operon</td>
</tr>
</tbody>
</table>

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.
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 C₄ 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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