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.
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\textsubscript{i} uptake genes in response to changing CO\textsubscript{2} conditions among *Microcystis* strains representative of the different C\textsubscript{i} uptake genotypes. Studies with *Synechocystis* PCC 6803 showed that genes for the high-affinity uptake systems for CO\textsubscript{2} (NDH-I\textsubscript{1}) and bicarbonate (SbtA and BCT1) are induced at low CO\textsubscript{2}, whereas the expression of low-affinity C\textsubscript{i} uptake systems (BicA and NDH-I\textsubscript{2}) remains unaltered (29, 30). It seems likely that the same expression patterns apply to *Microcystis*, although the presence of different C\textsubscript{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\textsubscript{i} uptake genes are downregulated at elevated CO\textsubscript{2} (1,100 ppm), whereas (ii) low-affinity C\textsubscript{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\textsubscript{2} levels. Furthermore, we measured O\textsubscript{2} evolution of the strains exposed to different CO\textsubscript{2} conditions to compare the activity of their C\textsubscript{i} uptake systems. The results reveal an unexpected diversity of CO\textsubscript{2} responses within the genus *Microcystis*.

**MATERIALS AND METHODS**

**Microcystis strains.** We studied six *Microcystis* strains with different C\textsubscript{i} uptake systems (Fig. 1). The C\textsubscript{i} uptake genotypes of these strains were described in a previous study (17). Strain PCC 7806 contains the bic\textsubscript{A} gene but lacks the sbt\textsubscript{A} gene and belongs to C\textsubscript{i} uptake genotype I. Strains NIES-843 and CCAP 1450/11 contain the sbt\textsubscript{A} gene but lack a complete bic\textsubscript{A} gene and hence belong to genotype II. Strain NIVA-CYA 140 combines sbt\textsubscript{A} with a complete bic\textsubscript{A} 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 bic\textsubscript{A} and sbt\textsubscript{A} 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 CO\textsubscript{2} uptake system NDH-I\textsubscript{3} (chpY, ndhD3, ndhF3, and other ndh genes) and the low-affinity CO\textsubscript{2} uptake system NDH-I\textsubscript{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\textsubscript{2} (400 ppm) and elevated pCO\textsubscript{2} (1100 ppm) in the gas flow to study gene expression and activity of the C\textsubscript{i} uptake systems.

First, the six *Microcystis* strains were precultured in 1-liter Erlenmeyer flasks in modified BG11 medium (34; with 10 mmol liter\textsuperscript{-1} NaNO\textsubscript{3} and no added Na\textsubscript{2}CO\textsubscript{3}/NaHCO\textsubscript{3}) for 1 week. The precultures were incubated at 25°C with 400 ppm of pCO\textsubscript{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\text{mol} photons m\textsuperscript{-2} s\textsuperscript{-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 (OD\textsubscript{750}) 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 pCO\textsubscript{2} 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 CO\textsubscript{2} fixation. Therefore, we decided to use low light levels of 20...
µmol photons m⁻² s⁻¹ during the batch culture experiments to minimize possible effects of microcytists 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 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 × 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-1095URI; Terumo Europe N.V., Leuven, Belgium), which were filled completely (using a needle to leave 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 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 1000 spectrophotometer (Thermo Scientific, San Jose, CA), and all RNA samples had A260/A280 and A260/A320 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: cmnA (encoding a subunit of the high-affinity bicarbonate transporter BCT1), bicA and sbtA (the two sodium-dependent bicarbonate transporters), chpX (dehydrogenase subunit of the low-affinity CO₂ uptake system NDH-1), chpY (dehydrogenase subunit of the high-affinity CO₂ uptake system NDH-1), 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 (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 C₄-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 OD₇50 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 lead 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]) 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 (Iᵣ) 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 (mg 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 CO2. Six *Microcystis* strains (PCC 7806, NIES-843, CCAP 1450/10, NIVA-CYA 140, HUB 5-3, and PCC 7005) with different C 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. 2and 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 supplementary material). The treatments in the O2 evolution experiments to study activity of the different C uptake systems are shown in Table 1. The dissolved inorganic carbon concentrations expected at pH 9.8 and 20.3°C are shown. The last column indicates which C uptake systems are mostly active at the applied conditions.

![FIG 2](image-url) 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](image-url) 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).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>KHCO₃ (μmol liter⁻¹)</th>
<th>NaCl (mmol liter⁻¹)</th>
<th>LiCl (mmol liter⁻¹)</th>
<th>CO2(aq) (μmol liter⁻¹)</th>
<th>HCO₃⁻ (μmol liter⁻¹)</th>
<th>Active Cᵢ 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>3.000</td>
<td>7891</td>
<td>NDH-I3, NDH-I4</td>
<td></td>
</tr>
</tbody>
</table>
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 ecaA) 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.

CO₂ uptake activity of low- and high-CO₂ acclimated cells. We studied O₂ evolution of the Microcystis strains to compare the activity of their CO₂ uptake systems when the strains were acclimated to low- or high-CO₂ conditions (Fig. 5). We applied six different treatments to activate different CO₂ 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-CO₂ 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

FIG 4 Heat map of changes in gene expression at elevated CO₂ 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 pCO₂ level from 400 to 1100 ppm. The color bar indicates log₂ values. Significant downregulated genes are shown in green, significant upregulated genes are shown in red, and nonsignificant changes (P > 0.05) or log₂ 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.
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 one-way analysis of variance with post hoc comparison of the means based on Tukey’s HSD test (α = 0.05).

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

For cells acclimated to high-Ci 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-Ci 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-Ci 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 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-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 Ci uptake systems in *Microcystis* and three model cyanobacterial strains

<table>
<thead>
<tr>
<th>Ci uptake system (genes involved)</th>
<th>Energy cost</th>
<th>Gene expression</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 HCO₃⁻</td>
<td>Inducible under low pCO₂</td>
<td>Inducible under low pCO₂</td>
<td>–</td>
<td>Inducible under low pCO₂</td>
</tr>
<tr>
<td>SbtA (<em>sbtA</em>)</td>
<td>0.5 ATP per HCO₃⁻</td>
<td>Constitutively expressed/inducible under low pCO₂/-</td>
<td>Inducible under low pCO₂</td>
<td>Inducible under low pCO₂</td>
<td>–</td>
</tr>
<tr>
<td>BicA (<em>bicA</em>)</td>
<td>0.25 ATP per HCO₃⁻</td>
<td>Constitutively expressed/inducible under low pCO₂/-</td>
<td>Constitutively expressed</td>
<td>Inducible under low pCO₂</td>
<td>–</td>
</tr>
<tr>
<td>NDH-I₃ (<em>chpY and others</em>)</td>
<td>1 NADPH per CO₂-to-HCO₃⁻ conversion</td>
<td>Constitutively expressed</td>
<td>Inducible under low pCO₂</td>
<td>Inducible under low pCO₂</td>
<td>Inducible under low pCO₂</td>
</tr>
<tr>
<td>NDH-I₄ (<em>chpX and others</em>)</td>
<td>1 NADPH per CO₂-to-HCO₃⁻ conversion</td>
<td>Constitutively expressed</td>
<td>Constitutively expressed</td>
<td>Constitutively expressed</td>
<td>Constitutively expressed</td>
</tr>
</tbody>
</table>

* The estimated energy costs of the different Ci 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 Ci uptake system is absent.

* 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 Ci 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 Ci 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 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 CO₂; none of them were upregulated. Hence, all Ci uptake systems that a strain was capable to produce were available for the cells at low CO₂ levels, including the low-affinity Ci 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-I₄ 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...
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 bicA and sbtA varied widely among the strains. Even similar genotypes, such as strains HUB 5-3 and PCC 7005 that take genes bicA and 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-C₄ conditions (Fig. 4D), whereas PCC 7005 mainly relies on ATP-dependent bicarbonate uptake by BCT1 (Fig. 4E). 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 therefore assigned to C₄ 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. 4C), whereas the strain depended mainly on CO₂ uptake under high-pCO₂ conditions (Fig. 4D). 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. 4B) 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-C₄ conditions, whereas genes encoding the low-affinity CO₂ uptake system NDH-I₃ 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 C₄ 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 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

| Table 3 Presence and function of the CCM transcriptional regulators in Microcystis and three model cyanobacterial strains |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Transcriptional regulator      | Location in genome | Function                           | Microcystis (17; this study) | Synechocystis PCC 6803 (29, 53) | Synechococcus PCC 7002 (50) | Synechococcus PCC 7942 (46, 54) |
| CcmR                            | Upstream of high-affinity CO₂ uptake operon | Repressor/activator of cmpABCD operon (BCT1) | Repressor of sbtA and high-affinity CO₂ uptake operon (not bicA) | Repressor of sbtA and bicA (possibly high-affinity CO₂ uptake operon) | – | – |
| CcmR2                           | Upstream of bicA-sbtA operon | Repressor/activator of bicA-sbtA operon | – | – | – | – |
| CmpR                            | Upstream of cmpABCD operon (BCT1) or separate location | Activator of cmpABCD operon | – | – | Activator of cmpABCD operon (possibly a repressor of sbtA and high-affinity CO₂ uptake operon) |

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.

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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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