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ORIGINAL ARTICLE

Genetic diversity of inorganic carbon uptake systems causes variation in CO_2 response of the cyanobacterium *Microcystis*

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Rising CO_2 levels may act as an important selective factor on the CO_2-concentrating mechanism (CCM) of cyanobacteria. We investigated genetic diversity in the CCM of *Microcystis aeruginosa*, a species producing harmful cyanobacterial blooms in many lakes worldwide. All 20 investigated *Microcystis* strains contained complete genes for two CO_2 uptake systems, the ATP-dependent bicarbonate uptake system BCT1 and several carbonic anhydrases (CAs). However, 12 strains lacked either the high-flux bicarbonate transporter BicA or the high-affinity bicarbonate transporter SbtA. Both genes, *bicA* and *sbtA*, were located on the same operon, and the expression of this operon is most likely regulated by an additional LysR-type transcriptional regulator (CcmR2). Strains with only a small *bicA* fragment clustered together in the phylogenetic tree of *sbtAB*, and the *bicA* fragments were similar in strains isolated from different continents. This indicates that a common ancestor may first have lost most of its *bicA* gene and subsequently spread over the world. Growth experiments showed that strains with *sbtA* performed better at low inorganic carbon (C_i) conditions, whereas strains with *bicA* performed better at high C_i conditions. This offers an alternative explanation of previous competition experiments, as our results reveal that the competition at low CO_2 levels was won by a specialist with only *sbtA*, whereas a generalist with both *bicA* and *sbtA* won at high CO_2 levels. Hence, genetic and phenotypic variation in C_i uptake systems provide *Microcystis* with the potential for microevolutionary adaptation to changing CO_2 conditions, with a selective advantage for *bicA*-containing strains in a high-CO_2 world.

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Introduction

High nutrient loads can favor the development of dense cyanobacterial blooms (Chorus and Bartram, 1999; Paerl, 2008; Brauer *et al.*, 2012), which increase the turbidity of lakes, deplete night-time oxygen concentrations, suppress the growth of submerged plants and thereby impair important underwater habitat for aquatic invertebrates and fish species (Scheffer, 1998; Gulati and van Donk, 2002). Moreover, several bloom-forming cyanobacteria produce toxins, causing serious and sometimes fatal liver, digestive and neurological diseases in birds and mammals, including humans (Carmichael, 2001; Cox *et al.*, 2003; Codd *et al.*, 2005). Cyanobacterial blooms are therefore a major threat to the use of lakes for drinking water, irrigation, fishing and recreation (Chorus and Bartram, 1999; Falconer, 2005; Huisman *et al.*, 2005). Although recent studies warn that cyanobacterial blooms may benefit from global warming (Jöhnk *et al.*, 2008; Paerl and Huisman, 2008; Kosten *et al.*, 2012; Michalak *et al.*, 2013), their possible response to rising CO_2 levels remains less clear.

Eutrophic lakes vary widely in the availability of inorganic carbon (C_i). Many lakes are supersaturated with CO_2 due to degradation of organic matter, sometimes resulting in dissolved CO_2 concentrations exceeding 10 000 p.p.m. (Cole *et al.*, 1994; Sobek *et al.*, 2005; Lazzarino *et al.*, 2009). However, the photosynthetic activity of dense cyanobacterial blooms can strongly deplete the dissolved CO_2 concentration, resulting in severely undersaturated CO_2 conditions (Ibelings and Maberly, 1998; Balmer and Downing, 2011; Gu *et al.*, 2011). CO_2 depletion by dense blooms raises the pH, sometimes even to pH > 10.5 (Talling, 1976; Jeppesen *et al.*, 1990;

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López-Archilla *et al*., 2004; Verschoor *et al*., 2013), shifting the C_i balance towards bicarbonate and carbonate.

Cyanobacteria have had a long evolutionary history, during which they have developed highly efficient CO_2-concentrating mechanisms (CCMs) that enable them to grow well at a wide range of C_i conditions (Kaplan and Reinhold, 1999; Giordano *et al*., 2005; Badger *et al*., 2006; Price *et al*., 2008; Raven *et al*., 2012). The CCMs of cyanobacteria are structurally and phylogenetically different from those of most eukaryotic algae (Raven, 2010; Wang *et al*., 2011b), and it has been argued that bloom-forming cyanobacteria are particularly adept to compete at the low CO_2 and high-pH conditions typical of dense blooms (Shapiro, 1990). Furthermore, several bloom-forming cyanobacterial species are buoyant and can float upwards, which provides an advantage in competition for light (Walsby *et al*., 1997; Huisman *et al*., 2004; Jöhnk *et al*., 2008) and enables surface blooms to intercept the CO_2 influx at the air – water interface (Paerl and Ustach, 1982; Ibelings and Maberly, 1998).

So far, five different C_i uptake systems have been identified in cyanobacteria, two for CO_2 (Shibata *et al*., 2001) and three for bicarbonate (Omata *et al*., 1999; Shibata *et al*., 2002; Price *et al*., 2004; Price, 2011). These uptake systems have different properties (Price *et al*., 2004), and not all uptake systems are present in every cyanobacterium (Rae *et al*., 2011). The CO_2 uptake systems convert CO_2, which has passively diffused into the cell, to bicarbonate in a NADPH-dependent reaction involving NDH-1 complexes with specific subunits (Price, 2011). The NDH-1_3 CO_2 uptake system has a high substrate affinity ($K_{0.5} = 1$–$2\ \mu M\ CO_2$) and low flux rate (Maeda *et al*., 2002; Price *et al*., 2002). Conversely, the NDH-1_4 CO_2 uptake system has a lower substrate affinity ($K_{0.5} = 10$–$15\ \mu M\ CO_2$) but high flux rate (Maeda *et al*., 2002; Price *et al*., 2002).

The three bicarbonate uptake systems are located in the plasma membrane (Price, 2011). The first, BicA, has a low substrate affinity ($K_{0.5} = 70$–$350\ \mu M$ bicarbonate, depending on the species) but a high flux rate (Price *et al*., 2004). The second bicarbonate transporter, SbtA, has a high substrate affinity ($K_{0.5} < 5\ \mu M$ bicarbonate) but low flux rate (Price *et al*., 2004). Downstream of *sbtA*, another associated gene, *sbtB*, is often found. The function of SbtB is still unknown, and it does not seem essential for SbtA activity (Price, 2011). Both SbtA and BicA are sodium-dependent bicarbonate transporters (Shibata *et al*., 2002; Price *et al*., 2004), although the molecular details of their sodium co-transport have not yet been elucidated (Price and Howitt, 2011; Price *et al*., 2011). The third bicarbonate transporter, BCT1, has a medium substrate affinity ($K_{0.5} = 10$–$15\ \mu M$ bicarbonate) and low flux rate (Omata *et al*., 2002). It consists of four subunits (CmpABCD) and is directly ATP dependent (Omata *et al*., 1999; Price *et al*., 2008).

In addition, cyanobacteria deploy a variety of CAs (Smith and Ferry, 2000; Badger *et al*., 2006). This includes periplasmic CAs, such as EcaA (α-type CA) and EcaB (β-type CA), which are thought to convert bicarbonate to CO_2 in the periplasmic space between the outer cell wall and plasma membrane to facilitate carbon transport into the cell (Soltes-Rak *et al*., 1997; So *et al*., 1998). The carboxysomal CcaA (β-type CA) converts bicarbonate to CO_2 in the carboxysome, thus enhancing the CO_2/O_2 ratio near the key enzyme RuBisCO to facilitate its CO_2 fixation activity.

In this paper, we analyze the genetic diversity of CCM genes among 20 different strains of the harmful cyanobacterium *Microcystis aeruginosa* (hereafter referred to as *Microcystis*). *Microcystis* is a widespread genus that can produce the toxin microcystin (MC) and develops dense blooms in many eutrophic lakes and brackish waters across the globe (Chen *et al*., 2003; Orr *et al*., 2004; Verspagen *et al*., 2006; Tonk *et al*., 2007; Paerl and Huisman, 2008; Michalak *et al*., 2013). Our analysis reveals genetic diversity in the presence/absence of the bicarbonate uptake genes *bicA* and *sbtA* among *Microcystis* strains. Moreover, we show that strains with *sbtA* grow better at low C_i conditions, whereas strains with *bicA* grow better at high C_i conditions. The presence of both genotypic and phenotypic variation in C_i uptake systems indicates that rising CO_2 levels may potentially affect the genetic composition of *Microcystis* blooms.

Materials and methods

Genome sequences

We investigated the CCM genes of 20 strains of *M. aeruginosa* (Kützing) (*sensu* Otsuka *et al*., 2001). Our analysis comprised 12 strains for which the full genome was sequenced in previous studies, including *Microcystis* NIES-843 (Kaneko *et al*., 2007), *Microcystis* PCC 7806 (Frangeul *et al*., 2008) and 10 *Microcystis* strains recently sequenced by Humbert *et al*. (2013).

In addition, we sequenced the entire genome of *Microcystis* PCC 7005 with the Illumina GAIIx platform (Baseclear, Leiden, The Netherlands). Paired-end sequencing was used with an insert size of 250 bp and a read length of 2×50 bp. This resulted in 27 800 092 reads, of which 94% was assembled with CLC bio's software version 4.7.2 into 1363 contigs with a total length of 4.9 Mb. The large number of contigs is due to many repeats and transposons, which are far more abundant in *Microcystis* than in most other cyanobacteria (Lin *et al*., 2011). This whole-genome shotgun sequencing project has been deposited at GenBank under the accession AQPY00000000. The version described in this paper is the first version, AQPY01000000.

We applied Blast (Altschul *et al.*, 1990) to search for CCM genes in the 12 previously sequenced *Microcystis* strains, using CCM gene sequences of *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 as reference. The genomic sequence data of strain PCC 7005 was investigated manually, using the CCM gene sequences of the other *Microcystis* strains as reference.

PCR analysis of additional strains

To enlarge our data set, we developed a series of PCR primers on the basis of the available genome sequences, to investigate the presence of specific CCM genes in seven additional *Microcystis* strains (CCAP 1450/10, CCAP 1450/11, HUB 5-2-4, HUB 5-3, NIVA-CYA 140, V145 and V163). The strains were maintained in our culture collection, using Erlenmeyer flasks incubated at 20 °C in BG11 medium (Rippka *et al.*, 1979) at ambient CO_2 and continuous light (~ 3 μmol photons $m^{-2} s^{-1}$). Samples for DNA isolation were pelleted by centrifugation (8000 *g*, 10 min at 20 °C). After five freeze – thawing cycles in liquid N_2 and at 65 °C, the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) was used to isolate total DNA according to the instructions for Gram-negative bacteria provided by the supplier.

PCR reactions to detect the presence of CCM genes were performed with the GoTaq DNA polymerase kit (Promega GmbH, Mannheim, Germany) according to the instructions of the supplier. The sequences of the new primers are listed in the Supplementary Information. The annealing temperature was 56 °C. Gel electrophoresis with ethidium bromide staining was used to analyze the PCR products. Selected PCR products of the sodium-dependent bicarbonate transporter genomic region in the seven *Microcystis* strains were sequenced by long run Quick Shot sequencing on an Applied Biosystems 3730XL sequencer (Baseclear). The sequence data were manually checked with the software program Chromas Lite, and overlapping sequences were combined. Most parts were sequenced in both directions. The sequences have been deposited in Genbank and are available under the following accession numbers: KC896021-KC896027.

Comparison of sequence data

The CCM gene sequences of the 20 investigated *Microcystis* strains were aligned with ClustalW version 2.1 (Thompson *et al.*, 1994) to investigate variation in transcription and translation potential as well as phylogenetic relationships among the different genotypes. Possible LysR transcriptional regulator binding sites were defined as TNA-$N_{7/8}$-TNA (Shively *et al.*, 1998; Woodger *et al.*, 2007; Nishimura *et al.*, 2008). The most likely −10 transcriptional start site was based on *Synechocystis* PCC 6803 data, most commonly 5′-TAAAAT-3′ (Mitschke *et al.*, 2011). Maximum likelihood phylogenetic trees of *bicA* and *sbtAB* were constructed with the software program MEGA version 5.05 (Tamura *et al.*, 2011) using 1000 bootstrap replicates.

Growth experiments

We investigated the specific growth rates of axenic strains of *Microcystis* NIES-843, PCC 7005 and PCC 7806, which represented three contrasting C_i uptake genotypes, in different C_i environments. A mutant of PCC 7806, incapable of MC production (PCC 7806 Δ*mcyB*; Dittmann *et al.*, 1997), was also tested to investigate possible effects of MC production. The experimental treatments comprised three different CO_2 levels in the gas flow (20 ± 1, 400 ± 20 and 10000 ± 400 ppm), with or without the addition of an alkaline buffer solution (5 mM CAPSO-NaOH, pH 10.0) to the mineral medium. Each treatment was replicated six times.

The growth experiments were performed in 24-well microplates (Corning Incorporated, New York, NY, USA) placed in three sterilized 1.7 L glass incubation chambers, which in turn were placed in a large Orbital incubator (Gallenkamp, Leicester, UK) that was maintained at 25 °C and provided 120 r.p.m. shaking and light from TL-D 30W/33-640 white fluorescent tubes (Philips, Eindhoven, The Netherlands). The photon flux density at the wells, measured with an LI-250 light meter (LI-COR Biosciences, Lincoln, NE, USA), was 14–16 μmol photons $m^{-2} s^{-1}$. The mineral medium was a modified BG11 medium (Rippka *et al.*, 1979) with 5 mM $NaNO_3$ and 5 mM NaCl, but without addition of $NaHCO_3$ or Na_2CO_3 ('BG11*'). The glass incubation chambers were provided with CO_2-enriched air at a flow rate of 25 L/h. The CO_2-enriched air was based on CO_2-depleted pressurized air (21% O_2, 78% N_2; CO_2 was removed by two 1 m high columns filled with NaOH pellets) to which different amounts of pure CO_2 gas were added, using the GT 1355R-2-15-A 316 SS Flow Controllers and 5850S Mass Flow Controllers (Brooks Instrument, Hatfield, PA, USA). The gas mixture was moistened with 25 °C water to suppress evaporation from the wells and led through a 0.20 μm Midisart 2000 filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) to sterilize the air, before it entered the incubation chambers. The CO_2 concentration in the gas mixture was checked regularly with an Environmental Gas Monitor for CO_2 (EGM-4; PP Systems, Amesbury, MA, USA).

At the start of the experiments, exponentially growing pre-cultures were diluted with BG11* to a similar absorbance at 750 nm of $A_{750} \approx 0.015$. The strains were randomized over the microplates to minimize position effects. The total start volume per well was 2200 μl. Samples of 100 μl were taken from the wells on an almost daily basis for at least 1 week and transferred in a sterile fume hood to 96-well microplates (Corning Incorporated) to measure the

A_{750} with a tunable Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA). A few wells close to the gas inlet of the incubation chambers showed substantial evaporation and were excluded from the data analyses. Specific growth rates were calculated from 3–5 time points during the exponential growth phase, as the slope obtained by linear regression of the natural logarithm of A_{750} versus time.

We ran an additional experiment with larger six-well microplates (Corning Incorporated), using the same experimental conditions, to gather sufficient sampling volume to characterize the C_i conditions induced by the treatments. We used three wells per treatment. The wells were inoculated with one representative strain, *Microcystis* PCC 7005, at $A_{750} \approx 0.015$, and sampled one day after inoculation. The pH in the wells was measured with a Lab 860 pH meter in combination with a BlueLine 28 Gel pH electrode (SCHOTT Instruments GmbH, Mainz, Germany). The well contents of the three biological replicates were combined, centrifuged at 4000 *g* for 10 min at 20 °C and filtered over 0.45 μm membrane filters (Whatman, Maidstone, UK) to determine the DIC concentration with a TOC-V_{CPH} analyzer (Shimadzu, Kyoto, Japan) using 3–5 technical replicates per analysis. Concentrations of dissolved CO_2 (aq), bicarbonate and carbonate were calculated from DIC and pH (Stumm and Morgan, 1996).

Growth data of each *Microcystis* strain were analyzed with a two-way analysis of variance to test whether the specific growth rates were affected by the CO_2 level in the gas flow and the alkaline buffer in the mineral medium. We used Type III Sum of Squares to account for unequal sample sizes and the presence of significant interaction terms. *Post hoc* comparisons of the means were based on Tukey's unequal N HSD test, using a significance level α of 0.001.

Results

CCM genes of Microcystis

Analysis of the CCM genes of the 20 investigated *Microcystis* strains revealed substantial genetic diversity in the sodium-dependent bicarbonate uptake genes *bicA* and *sbtA* (Table 1). In total, 8 strains contained both *bicA* and *sbtA*, 1 strain (NIVA-CYA 140) had a transposon inserted in a complete *bicA* gene, 10 strains contained a small fragment of *bicA* but lacked a complete *bicA* gene and 1 strain (PCC 7806) lacked the *sbtAB* genes. Genes encoding the two CO_2 uptake systems Ndh-1_3 and Ndh-1_4, the ATP-dependent bicarbonate uptake system BCT1 and the three CAs EcaA, EcaB and CcaA were detected in all 20 strains. Strains NIES-843, PCC 9443, PCC 9807 and PCC 9809 contained an additional but somewhat shorter copy of *ccaA*, called *ccaA2*, next to the original gene. We found β-carboxysomal structural genes in all 13 fully sequenced *Microcystis* strains; these were not further investigated by PCR in the additional seven *Microcystis* strains. There was no relationship between the presence of C_i uptake system genes and MC genes (Table 1).

Variation in sodium-dependent bicarbonate uptake genes

Whenever present, *bicA* and *sbtA* were located in the same genomic region and were flanked by two genes encoding a LysR-type transcriptional regulator and a sodium/proton antiporter NhaS3, which most likely removes sodium imported by the sodium-dependent bicarbonate uptake systems. We distinguished three genotypes (Figure 1): genotype I contains *bicA* without *sbtAB,* genotype II contains *sbtAB* without (complete) *bicA* and genotype III contains both *bicA* and *sbtAB*. The three genotypes were further subdivided depending on differences in intergenic sequences and the presence of *bicA* fragments or transposons. Strain PCC 9443 was not included in Figure 1, because part of its sequence data between the *bicA* fragment and *sbtA* are missing but most likely this strain has genotype IIB or IIC.

Transcript analysis showed that *bicA*, *sbtAB* and the sodium/proton antiporter gene *nhaS3* were co-transcribed (see Supplementary Information), which indicates that they are probably located on the same operon. The upstream LysR-type transcriptional regulator gene (in reverse orientation) has thus far not been described for other cyanobacteria but appeared to be very similar to *ccmR*, which encodes for the transcriptional regulator of another CCM gene domain with the high-affinity CO_2 uptake genes. Therefore, we have called it *ccmR2* and it is anticipated to regulate the transcription of the *bicA-sbtAB-nhaS3* operon. An amino acid alignment revealed high similarities between the different CcmR-like transcriptional regulators in *Microcystis* (see Supplementary Information for details). There was some variation in CcmR2 between different strains, especially at the C-terminus. However, we did not find a relation between the C_i uptake genotypes and the sequence of CcmR2.

Phylogenetic analysis of bicA and sbtA

Phylogenetic trees were constructed on the basis of the DNA sequences of *bicA* and *sbtAB* (Figure 2). The strains showed more variation in *sbtAB* than in *bicA*. Clustering of the strains in the phylogenetic tree of *sbtAB* was largely based on the presence or absence of *bicA*. An exception was HUB 5-2-4, which lacked *bicA* but clustered with the strains containing both *sbtAB* and *bicA*. In addition, strain PCC 9806 and three strains from Africa and Australia formed outgroups. Strains that were toxic or isolated from proximate locations did not group together in the phylogenetic trees. Yet, there seems

Table 1 The presence of several CCM genes in selected reference cyanobacteria and the investigated *Microcystis* strains

Cyanobacterium	*Strain origin*	*C_i uptake systems*					*Carbonic anhydrases*			*MC genes*
		CO_2		*HCO_3^-*			α	*β*		
		Ndh-1$_3$[a]	*Ndh-1$_4$*[b]	*BicA*[b]	*SbtA*[a]	*BCT1*[a]	*EcaA*	*EcaB*	*CcaA*	
Synechocystis PCC 6803	USA (California)	+	+	2	+	+	−	+	+	−
Synechococcus PCC 7002	PRI (Magueyes Island)	+	+	2	+	−	+	−	+	−
Synechococcus PCC 7942	USA (Texas)	+	+	−	+	+	+	−	+	−
Anabaena ATCC 29413	USA (Mississippi)	+	+	+, ≠	+	+	+	2	+	−
Microcystis CCAP 1450/10[c]	GBR (Lake Blelham Tarn)	+	+	≠	+	+	+	+	+	+
Microcystis CCAP 1450/11[c]	JPN (Tokyo)	+	+	≠	+	+	+	+	+	−
Microcystis HUB 5-2-4[c]	DEU (Lake Pehlitzsee)	+	+	≠	+	+	+	+	+	+
Microcystis HUB 5-3[c]	DEU (Lake Pehlitzsee)	+	+	+	+	+	+	+	+	−
Microcystis NIES-843	JPN (Lake Kasumigaura)	+	+	≠	+	+	+	+	2	+
Microcystis NIVA-CYA 140[c]	CAN (Lake Bruno)	+	+	#	+	+	+	+	+	+
Microcystis PCC 7005	USA (Lake Mendota)	+	+	+	+	+	+	+	+	−
Microcystis PCC 7806	NLD (Braakman)	+	+	+	−	+	+	+	+	+
Microcystis PCC 7941	CAN (Little Rideau Lake)	+	+	+	+	+	+	+	+	+
Microcystis PCC 9432	CAN (Little Rideau Lake)	+	+	+	+	+	+	+	+	−
Microcystis PCC 9443	CAF	+	+	≠	+	+	+	+	2	+
Microcystis PCC 9701	FRA (Guerlesquin dam)	+	+	≠	+	+	+	+	+	−
Microcystis PCC 9717	FRA (Rochereau dam)	+	+	≠	+	+	+	+	+	−
Microcystis PCC 9806	USA (Oshkosh)	+	+	+	+	+	+	+	+	−
Microcystis PCC 9807	ZAF (Hartbeespoort dam)	+	+	+	+	+	+	+	2	+
Microcystis PCC 9808	AUS (Malpas dam)	+	+	≠	+	+	+	+	+	+
Microcystis PCC 9809	USA (Lake Michigan)	+	+	+	+	+	+	+	2	+
Microcystis T1-4	THA (Bangkok)	+	+	≠	+	+	+	+	+	−
Microcystis V145[c]	NLD (Lake Volkerak)	+	+	+	+	+	+	+	+	−
Microcystis V163[c]	NLD (Lake Volkerak)	+	+	≠	+	+	+	+	+	+

≠, only a small fragment of the gene is present; #, the gene is complete but disrupted by a transposon; 2, the genome contains two copies of the gene. The origins of the strains are indicated with three-letter codes of the different countries (ISO 3166-1 α-3). The presence of CCM genes is based on high similarity with the reference genes in *Synechocystis* PCC 6803 or *Synechococcus* PCC 7942. The presence of microcystin (MC) genes indicates potentially toxic strains.
[a]C_i uptake system with a high substrate affinity and low flux rate.
[b]C_i uptake system with a low substrate affinity and high flux rate.
[c]For these strains, results are based on PCR product detection with primers for the concerned gene; PCR products of *sbtA* and *bicA* of these *Microcytis* strains were sequenced.

to be some large-scale biogeographical pattern, as all the six North-American strains contained both *sbtAB* and *bicA* (but with a transposon insert in *bicA* of NIVA-CYA 140), whereas all the three Asian strains lacked a complete *bicA*. Strains from Europe included both genotypes.

Growth experiments

In the growth experiments, higher CO_2 levels in the gas flow yielded higher concentrations of dissolved CO_2 (Figure 3a), higher bicarbonate concentrations (Figure 3b) and a lower pH (Figure 3c) in the mineral medium. The addition of CAPSO buffer increased the alkalinity, which resulted in a higher bicarbonate concentration and in a higher pH at 400 and 10 000 p.p.m. CO_2.

The specific growth rate of strain PCC 7806, which contained *bicA* but lacked *sbtA*, was significantly reduced at the lowest CO_2 level of 20 p.p.m., especially in the absence of the CAPSO buffer (Figure 4a; Supplementary Table S3 of the Supplementary Information). Addition of CAPSO buffer at 20 p.p.m. CO_2 increased the bicarbonate concentration and significantly improved the growth rate of PCC 7806, although it remained below the growth rates at 400 and 10 000 p.p.m. CO_2. The PCC 7806 Δ*mcyB* mutant responded similarly as the wild type, indicating that MC production had little or no effect on the CO_2 response in this study (Figure 4b).

Strain PCC 7005, which contained both *bicA* and *sbtA*, maintained a high growth rate irrespective of the applied CO_2 level and CAPSO buffer (Figure 4c).

The growth rate of strain NIES-843, which contained *sbtA* but lacked a complete *bicA* gene, was significantly reduced at the highest CO_2 level of 10 000 p.p.m., especially in the absence of CAPSO buffer (Figure 4d). Addition of CAPSO buffer at 10 000 p.p.m. CO_2 significantly improved the growth rate of NIES-843, although it remained below the growth rates at 20 and 400 p.p.m. CO_2.

Discussion

CCM genes of Microcystis

Our results show genetic diversity among the C_i uptake systems of *Microcystis*. In particular, the presence of the sodium-dependent bicarbonate

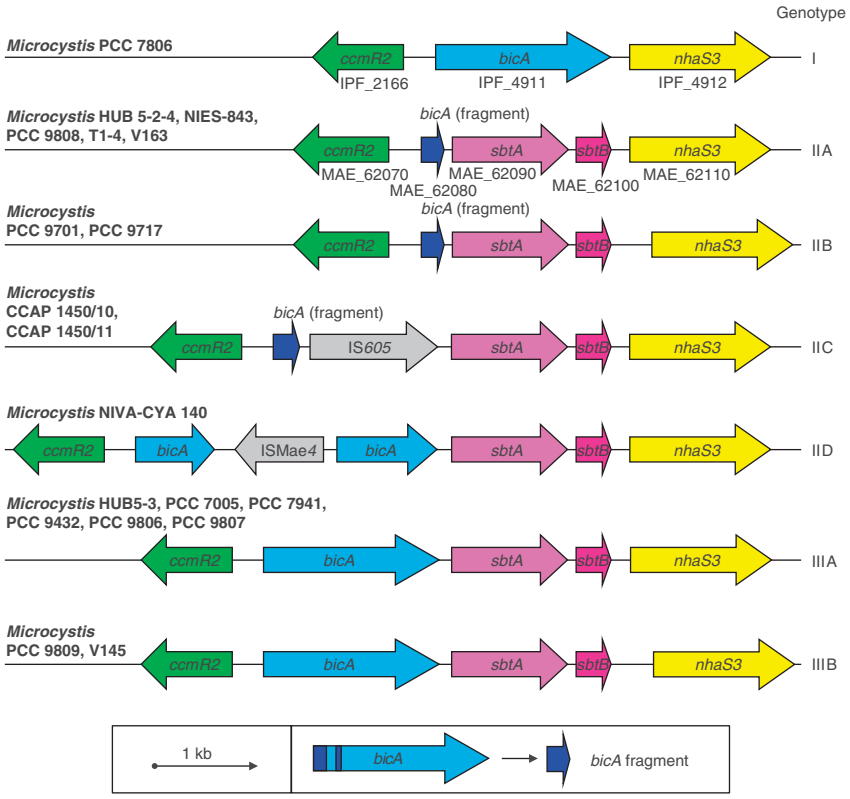

Figure 1 Variation of the sodium-dependent bicarbonate uptake genomic region in different *Microcystis* strains. The genomic regions contain the sodium-dependent bicarbonate uptake genes (*bicA* and *sbtAB*) and adjacent genes encoding a transcriptional regulator (*ccmR2*) and a sodium/proton antiporter (*nhaS3*). Several strains lack a complete *bicA* but have a *bicA* fragment consisting of two parts of the 5′-end of *bicA* (indicated in the bottom panel). NIVA-CYA 140 has a defective *bicA* gene caused by a transposon insertion (ISMae*4*). CCAP 1450/10 and 1450/11 have a transposon insert (IS*605*) next to a *bicA* fragment. Adjacent genes upstream of *ccmR2* are *CP12* (Calvin–Benson cycle protein) or *ftsH* (cell division factor). Adjacent genes downstream of *nhaS3* include *N6amt1* (N6-adenine-specific DNA methyltransferase type I), a gene encoding for a 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase and hypothetical genes. The IPF numbers are the locus tags of PCC 7806, and the MAE numbers are the locus tags of NIES-843. The size marker indicates 1 kb.

uptake genes *bicA* and *sbtA* varied among the strains, which confirms an earlier suggestion that *Microcystis* strains might differ in their bicarbonate uptake systems (Bañares-España *et al*., 2006). We found 8 strains with both *bicA* and *sbtA*, 11 strains with a small fragment or a defected *bicA* but the complete *sbtA*, whereas 1 strain had a complete *bicA* but lacked *sbtA*.

On the basis of the available sequencing data, other cyanobacterial species containing both *bicA* and *sbtA* seem to have these genes located in different genomic regions. However, *Microcystis* showed a common genome domain for *bicA*, *sbtA*, *sbtB* and the sodium/proton antiporter gene *nhaS3* (Figure 1). Transcript analysis revealed that these genes were co-transcribed (see Supplementary Information). Hence, to our knowledge, *Microcystis* is the first example of a cyanobacterium in which *bicA* and *sbtA* are located on the same operon. The presence of a sodium/proton antiporter gene on the same operon strengthens the hypothesis (Shibata *et al*., 2002; Price *et al*., 2004) that bicarbonate transport by BicA and SbtA is sodium-dependent. The presence of an upstream LysR-type transcriptional regulator gene, *ccmR2*, makes it likely that transcription of the *bicA-sbtAB-nhaS3* operon is strongly regulated and dependent on environmental conditions.

Genes encoding the two CO_2 uptake systems, NDH-1_3 and NDH-1_4, were present in all 20 investigated *Microcystis* strains. Hence, our results support earlier suggestions (Price *et al*., 2008; Rae *et al*., 2011) that both CO_2 uptake systems are widespread among freshwater cyanobacteria. Similarly, all 20 *Microcystis* strains contained the *cmpABCD* genes encoding the bicarbonate uptake system BCT1. We did not find genes with homology to the transcriptional regulator gene *cmpR* of *Synechocystis* and *Synechococcus* in any of the 20 *Microcystis* strains (see Supplementary Information). Most likely, however, the expression of the *cmpABCD* operon and the high-affinity CO_2 operon are strongly regulated, as we detected potential LysR transcriptional regulator binding sites near the most likely −10 TSS of both operons, and found the LysR transcriptional regulator gene *ccmR* upstream of the high-affinity CO_2 operon.

It has been suggested that the two periplasmic CAs, EcaA and EcaB, are not widespread among

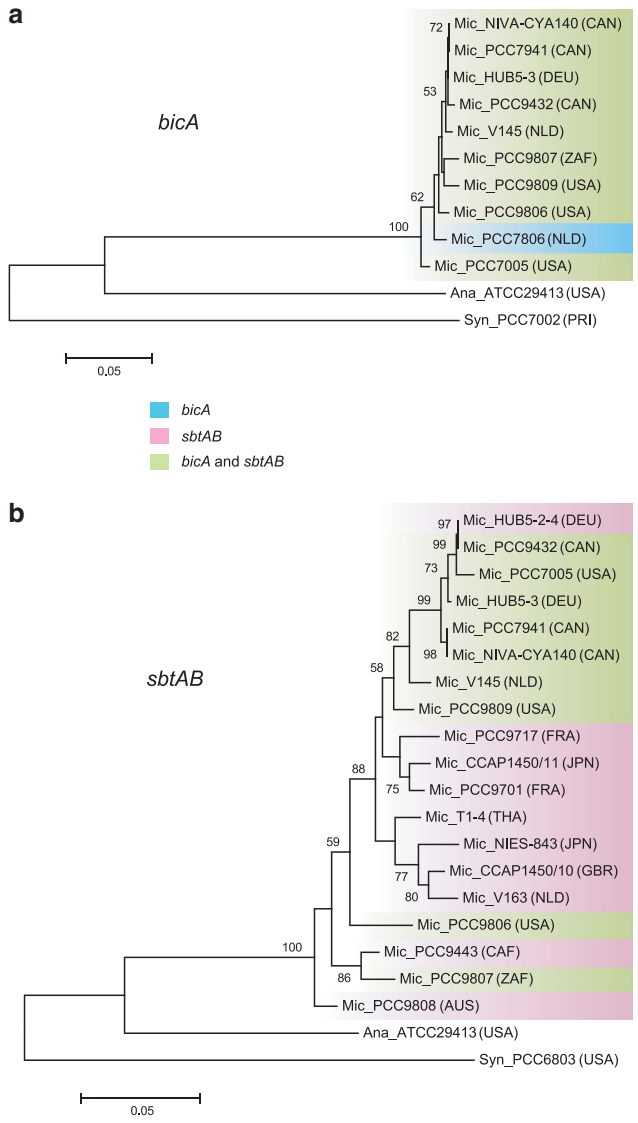

Figure 2 Phylogenetic trees of the sodium-dependent bicarbonate uptake genes of *Microcystis*. (**a**) Phylogenetic tree based on the *bicA* sequences. The outgroup cyanobacteria are *Anabaena variabilis* ATCC 29413 and *Synechococcus* PCC 7002, which both have complete *bicA*. (**b**) Phylogenetic tree based on the *sbtAB* sequences. The outgroup cyanobacteria are *Anabaena variabilis* ATCC 29413 and *Synechocystis* PCC 6803, which both have complete *sbtAB*. Color coding indicates the presence of *bicA* and *sbtAB* in the different *Microcystis* strains. The origin of the strains is shown with three-letter codes indicating the different countries (ISO 3166-1 α-3). The NIVA-CYA 140 *bicA* sequence consists of the two parts originally separated by a transposon insert (Figure 1). Bootstrap values (1000 replicates) are shown for confidence levels higher than 50%. The scale bars indicate the number of nucleotide substitutions per site.

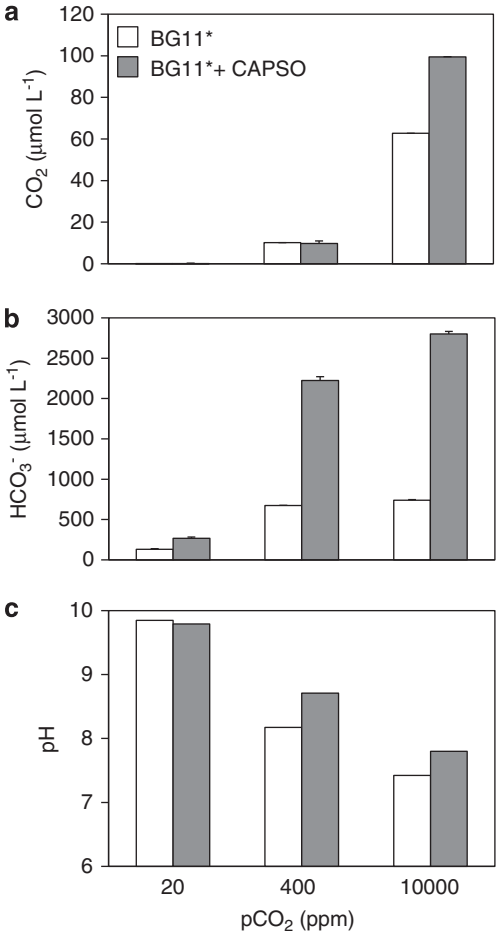

Figure 3 Dissolved C_i and pH in the growth experiments. (**a**) Dissolved CO_2 concentration, (**b**) bicarbonate concentration and (**c**) pH in growth experiments with strain PCC 7005 in six-well microplates after 1 day of incubation. Dissolved CO_2 and bicarbonate concentrations were calculated from measurements of pH and DIC in the combined supernatant of three biological replicates. Error bars in (**a**) and (**b**) represent the s.d. on the basis of 3–5 repeated measurements in the combined supernatant.

cyanobacteria (Badger *et al.*, 2006) and do not have an essential role in the CCM of cyanobacteria (So *et al.*, 1998). However, we found both periplasmic CA genes in all 20 *Microcystis* strains. Similarly, Wu *et al.* (2011) detected transcripts of both *ecaA* and *ecaB* in all their eight *Microcystis* strains from Chinese lakes. Hence, at least in *Microcystis*, these periplasmic CAs are very common.

The carboxysomal CA, CcaA contributes to the delivery of CO_2 to RuBisCO and hence has an important role in the CCM of cyanobacteria (So *et al.*, 1998). Indeed, *ccaA* was present in all our 20 strains as well as the 8 *Microcystis* strains investigated by Wu *et al.* (2011). Only 4 of our 20 strains contained both *ccaA* and *ccaA2*. The amino-acid sequence of CcaA2 is slightly shorter than CcaA, and the two different isoforms might enable cells to modify their CO_2 fixation efficiency (Long *et al.*, 2007), although further confirmation of this hypothesis is warranted.

Phylogenetic analysis of bicA and sbtAB

European and Asian strains with a complete *sbtAB* but only a *bicA* fragment clustered together in the phylogenetic tree of *sbtAB* (Figure 2). It is therefore likely that their ancestors first lost a large part of *bicA* and subsequently spread over the world.

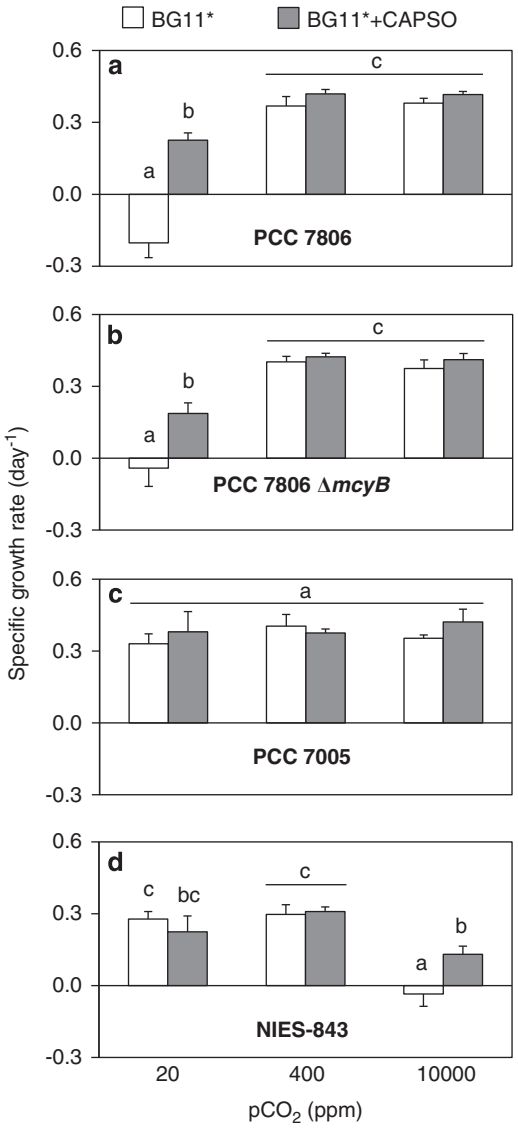

Figure 4 Specific growth rates of *Microcystis* strains representative of the three different C_i genotypes at different CO_2 levels. (**a**) Strain PCC 7806, which contains only *bicA*, (**b**) the mutant strain PCC 7806 Δ*mcyB*, which contains only *bicA* and lacks MC production, (**c**) strain PCC 7005, which contains both *bicA* and *sbtA* and (**d**) strain NIES-843, which contains only *sbtA*. Error bars indicate the s.d. of 3–6 biological replicates. For each strain, bars with different letters indicate significant differences in growth rate, as tested by a two-way analysis of variance with *post hoc* comparison of the means (see Supplementary Information for details).

Other evidence for a common *bicA* history is that the detected small *bicA* fragments were very similar in these Eurasian strains, the African strain PCC 9443 and the Australian strain PCC 9808. The *bicA* fragments consisted of two short sequences at the 5′-end part of *bicA* (inset in Figure 1), whereas the area missing in between has the HIP1 sequences (5′-CGATCG-3′) at the outer ends, which have been associated with early gene deletion events (Robinson *et al.*, 1995, 1997). One exception was HUB 5-2-4, which contained the same *bicA* fragment but clustered together in the *sbtAB* tree with strains that had complete *bicA*. Both the *sbtAB* and *nhaS3* genes of HUB 5-2-4 were very similar to PCC 9432. Possibly HUB 5-2-4 acquired these two genes from a recombination event with PCC 9432 or closely related strain, because other gene sequences (*cpcBA*, *ccmR2*) of HUB 5-2-4 and PCC 9432 were less similar.

At first sight, clustering in the phylogenetic trees shows little relationship with the geographical locations from which the strains were collected (Figure 2). This is in agreement with Janse *et al.* (2004) and Van Gremberghe *et al.* (2011), who did not find a relationship between the rDNA internal-transcribed spacer sequences of *Microcystis* strains and their geographical location, suggesting global dispersal of *Microcystis* strains. However, our six North-American strains contained both *sbtAB* and *bicA* (with a transposon insert in *bicA* of NIVA-CYA 140), the three Asian strains lacked complete *bicA*, strains from Europe included both genotypes and the three strains from Africa and Australia formed outgroups in the *sbtAB* tree. Further studies will be required to verify and explain such possible biogeographical differences.

Growth under different C_i conditions

Our growth experiments support earlier evidence that the sodium-dependent bicarbonate uptake systems BicA and SbtA can have a major impact on cyanobacterial growth (Shibata *et al.*, 2002; Xu *et al.*, 2008). Strains PCC 7005 and NIES-843 both contained the high-affinity but low-flux bicarbonate uptake system SbtA. They grew well at 20 p.p.m. CO_2, presumably because SbtA enabled them to make use of low bicarbonate availability. In contrast, strain PCC 7806 lacked SbtA and was unable to reach a positive growth rate at 20 p.p.m. CO_2 in the absence of CAPSO. Addition of CAPSO buffer at 20 p.p.m. CO_2 increased the bicarbonate concentration (Figure 3b) and thereby enhanced the growth rate of PCC 7806.

Strain PCC 7806 did contain the ATP-dependent bicarbonate uptake system BCT1, which has a medium affinity for bicarbonate. Apparently, the presence of BCT1 could not compensate for the lack of SbtA. This supports the view, based on mutants without a functional BCT1, that BCT1 most likely has a minor role in C_i uptake (Shibata *et al.*, 2002). However, an alternative explanation might be that the relatively high sodium concentration in our growth experiments may have favored the sodium-dependent bicarbonate transporters relative to BCT1. As *Microcystis* occur in both freshwater and brackish environments (Tonk *et al.*, 2007), it would be interesting to further investigate the role of sodium in the bicarbonate uptake of *Microcystis* in detail.

Strains PCC 7806 and PCC 7005 both contained the low-affinity but high-flux bicarbonate uptake

system BicA. They grew equally well at 400 and 10 000 p.p.m. CO_2, despite the differences in pH generated by the two applied CO_2 concentrations. BicA most likely enabled them to profit from the high bicarbonate concentrations. In contrast, strain NIES-843 lacked BicA and showed impaired growth at 10 000 p.p.m. CO_2. Possibly, this very high CO_2 level reduced its *sbtA* expression, which may have negative effects on growth when *bicA* is lacking. It might also be that the high CO_2 level reduced the pH to such an extent (Figure 3c) that the growth rate of NIES-843 was negatively affected, because some *Microcystis* strains are sensitive to $pH < 8$ (Wang *et al.*, 2011a). Addition of CAPSO buffer raised the pH and improved the growth of NIES-843 at 10 000 p.p.m. CO_2.

Our results show that *Microcystis* strains that produce both BicA and SbtA, such as strain PCC 7005, are most versatile in their growth response to different C_i conditions. Hence, why would several strains have lost *bicA* while some others lack *sbtA*? Possibly, the production of two partially redundant bicarbonate transporters is relatively costly, giving a selective disadvantage when only one of the two transporters is needed. If so, C_i uptake generalists (with both *bicA* and *sbtA*) are expected to lose the competition against high-affinity specialists (only *sbtA*) at low C_i conditions and against high-flux specialists (only *bicA*) at high C_i conditions.

Microcystis blooms and rising CO_2

Interestingly, our results reveal that *Microcystis* blooms may consist of mixtures of strains with different C_i uptake systems. Strains V145 and V163 were isolated from the same *Microcystis* bloom in Lake Volkerak, The Netherlands (Kardinaal *et al.*, 2007). However, although strain V145 contained all five C_i uptake systems, strain V163 lacked a complete *bicA* (Table 1). Similarly, strains HUB 5-2-4 and HUB 5–3 were isolated from the same *Microcystis* bloom in Lake Pehlitzsee, Germany (Schwabe *et al.*, 1988), but strain HUB 5–3 contained all five C_i uptake systems, whereas strain HUB 5-2-4 lacked *bicA*.

Genetic variation in C_i uptake systems may offer an important template for natural selection. During the development of cyanobacterial blooms, the amount of C_i available per cell is often gradually reduced, which may ultimately result in severely C_i-depleted conditions with a high pH (Ibelings and Maberly, 1998; Balmer and Downing, 2011). This might shift the selective advantage towards strains with *sbtA*, which perform better at low C_i conditions than strains lacking *sbtA* (Figure 4). Similarly, the evolutionary loss of *bicA* in several *Microcystis* strains might be explained by natural selection during periods of low C_i availability. Conversely, rising atmospheric CO_2 concentrations may enhance the C_i availability in lakes, alleviating cyanobacterial blooms from carbon limitation (Schippers *et al.*, 2004). This would disfavor high-affinity C_i uptake systems (Collins *et al.*, 2006), whereas strains with *bicA* will have a selective advantage in a high-CO_2 world.

These hypotheses are at least partially confirmed by the recent competition experiments of Van de Waal *et al.* (2011) highlighted by Wilhelm and Boyer (2011). They found that the toxic *Microcystis* strain NIVA-CYA 140 displaced the non-toxic strain NIVA-CYA 43 (=PCC 7005) at low CO_2 conditions, whereas the outcome of competition was reversed at high CO_2 conditions. On the basis of additional experiments with *Microcystis* PCC 7806 and its non-MC-producing mutant, Van de Waal *et al.* explained their results by differences in MC production between the strains, implicating that MCs might have a role in carbon assimilation (see also Zilliges *et al.*, 2011). However, in our study, we did not find an association between C_i uptake genotypes and MC genes. Furthermore, we did not find differences in CO_2 response between the MC-producing wild-type PCC 7806 and its non-MC-producing mutant. Instead, our findings suggest that the results of Van de Waal *et al.* (2011) can be more parsimoniously explained as a shift in competitive dominance from a high-affinity specialist with only *sbtA* (NIVA-CYA 140) at low CO_2 levels to a C_i uptake generalist with *bicA* and *sbtA* (PCC 7005) at high CO_2 levels.

Some words of caution are in place for the extrapolation of these laboratory results to natural field conditions. Our laboratory experiments and the competition studies of Van de Waal *et al.* (2011) were designed to investigate effects of different C_i conditions. We used relatively low light levels, whereas in reality *Microcystis* often performs vertical migrations during blooms, experiencing light conditions ranging from near-zero light levels deeper down in the water column to high light levels of 1500–2000 μmol photons $m^{-2} s^{-1}$ at the water surface (Visser *et al.*, 1997; Wallace *et al.*, 2000). Furthermore, in the real world, bloom-forming cyanobacteria compete not only with each other but also with numerous eukaryotic algal species, and their competitive success depends on a multitude of environmental factors including nutrient and light availability, temperature, turbulence, residence time, grazing and viruses (Dokulil and Teubner, 2000; Huisman *et al.*, 2005; Jöhnk *et al.*, 2008; Paerl, 2008). Yet, bloom-forming cyanobacteria do experience extensive variation in environmental C_i concentrations, including strong CO_2 depletion during dense blooms. The presence of genetic and phenotypic variation in C_i uptake systems of *Microcystis* is therefore likely to be of ecological relevance. Hence, our laboratory results clearly call for field studies investigating changes in gene expression and genotype composition of the C_i uptake systems during cyanobacterial bloom development.

In conclusion, we found genetic variation in the C_i uptake systems of *Microcystis* that was reflected by phenotypic variation in CO_2 response and that

explained the selection of strains with different C_i uptake strategies at different CO_2 levels. These laboratory results indicate that rising atmospheric CO_2 levels may in potential induce changes in the genotype composition of *Microcystis*, one of the major bloom-forming cyanobacteria threatening the water quality of many lakes and estuaries worldwide. Future field studies may wish to investigate whether and to what extent this potential for microevolutionary change is realized.

Conflict of Interest

The authors declare no conflict of interest.

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