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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Microcystin-degrading bacteria affect *mcyD* expression and microcystin synthesis in *Microcystis* spp.

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ARTICLE INFO

Article history:
Received 2 March 2015
Revised 17 June 2015
Accepted 20 June 2015
Available online 23 October 2015

Keywords:
Microcystis
Microcystins
MC-degrading bacteria
mcyD gene

ABSTRACT

Cyanobacterial blooms occur increasingly often and cause ecological, economic and human health problems worldwide. Microcystins (MCs) are the dominant toxins produced by cyanobacteria and are implicated in epidemic disease and environmental problems. Extensive research has been reported on the various regulating factors, *e.g.*, light, temperature, nutrients such as nitrogen and phosphorus, pH, iron, xenobiotics, and predators, that influence microcystin (MC) synthesis, but little is known about the effects of cyanobacteria-associated bacteria on MC synthesis. A considerable number of studies have focused on interactions between *Microcystis* species and their associated bacteria. In this study, we evaluated the effects of MC-degrading bacteria (MCDB) on MC synthesis gene *mcyD* expression and MC synthesis in axenic strain PCC7806, non-axenic strain FACHB905, and colony strain FACHB1325 of *Microcystis* by quantitative real-time polymerase chain reaction (RT-PCR) assay and enzyme-linked immunosorbent assay (ELISA). We demonstrate for the first time that MCDB can induce and up-regulate the MC production and transcriptional response of the *mcyD* gene of toxic *Microcystis*. On day 4 of the culturing experiment, the intracellular MC concentration and transcriptional response of *mcyD* of FACHB1325 were up-regulated 1.9 and 5.3-fold over that of the control, and for FACHB905 were up-regulated 1.8 and 4.2-fold over that of the control, respectively. On day 10, the transcriptional response of *mcyD* was up-regulated 21.3-fold in PCC7806. These results indicate that there are interactions between toxic *Microcystis* and MCDB, and MCDB may play a role in regulating *mcyD* expression in toxic *Microcystis*.

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Introduction

In recent decades, toxin-producing cyanobacteria harmful algal blooms (CyanoHABs) in freshwater ecosystems, caused by nutrient over-enrichment (eutrophication) and climate-change effects (the greenhouse effect), have become frequent and problem around the globe (Paerl and Otten, 2013). These blooms can consume dissolved oxygen and produce a broad range of toxic, bioactive secondary metabolites that kill aquatic plants, invertebrates, and fish in lakes throughout the world and also have a harmful effect on humans, animals, and other eukaryotic organisms (Falconer, 1999).

Toxic *Microcystis* cells possess a suite of microcystin (MC) synthesis genes (*mcyA-mcyJ*), while non-toxic strains do not

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http://dx.doi.org/10.1016/j.jes.2015.06.016
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(Davis et al., 2009). Microcystins (MCs), of which more than 90 different structural variants have been identified, are found ubiquitously worldwide (Ufelmann et al., 2012).

Interactions between cyanobacteria and associated bacteria have been intensively studied (Christoffersen et al., 2002; Eiler and Bertilsson, 2004; Grossart et al., 2006; Grossart and Simon, 2007), including the following four major modes: (1) bacteria and cyanobacteria form symbioses, in which bacteria benefit from phytoplankton exudates and cyanobacterial growth is favored by bacterial products such as vitamins, remineralized nutrients, and other growth factors; (2) bacteria act as parasites on phytoplankton and, therefore, can lead to lysis and death of their hosts, while cyanobacteria can also inhibit bacterial growth by releasing antibiotic compounds; (3) commensalistic bacteria have no actual negative or positive effects on phytoplankton, but the transition between commensalism and parasitism is highly variable over time; and (4) bacteria are only loosely associated with phytoplankton, and thus can efficiently compete for limiting nutrients such as phosphate.

MCs are produced in and excreted from healthy cyanobacterial cells. Before reaching the stationary phase, approximately 10% to 20% of MCs are lost from healthy cyanobacterial cells in culture (Sivonen, 1990; Rapala et al., 1997). When *Microcystis* cells decay, MCs are released from the cells, so the concentration of dissolved MCs can increase (Watanabe et al., 1992). In many freshwater lakes, MC concentrations have been reported to exceed guideline levels and thus cause widespread and serious threat to public health and ecosystem functioning (Chorus et al., 2001; Codd et al., 2005). A large number of studies have been published on the natural routes of MC detoxification. Many researchers have proposed that MCs in laboratory and field experiments are mainly degraded by co-existing microorganisms (Jones and Orr, 1994; Cousins et al., 1996; Bourne et al., 1996). But the mechanism of *in situ* degradation of MC remained to be clarified until Jones et al. (1994) isolated the first MC-degrading bacteria (MCDB), *Sphingomonas* sp. MJ-PV, from Australian water bodies.

It has been reported that many environmental factors and some zooplankton such as cladocerans and copepods can influence the synthesis of MCs in *Microcystis* species. For example, high light intensity resulted in an increase of transcription of *mcyB* and *mcyD* (Kaebernick et al., 2000). Nitrogen (nitrate and ammonium) and phosphorus limitation were also found to up-regulate the transcription of *mcyD* (Pimentel and Giani, 2014). Iron deficient conditions resulted in an increase of *mcyD* and *mcyH* transcription, correlating with an increase in microcystin-leucine-arginine (MC-LR) levels (Sevilla et al., 2008; Alexova et al., 2011). Pyrogallol stress, a potent allelochemical, up-regulated *mcyB* gene expression in *Microcystis* (Shao et al., 2009). On consumption by *Daphnia*, *mcyA* gene expression was up-regulated in *Microcystis* and production of MCs increased (Pineda-Mendoza et al., 2014).

Previous work in our laboratory showed that the MC concentration in field water may affect communities of MCDB; toxic *Microcystis* and MCDB have both direct and indirect influences on each other (Zhu et al., 2014). However, little information is available regarding the mechanisms of the interactions between MCDB and toxic *Microcystis*. The aim of this study was to measure changes in *mcyD* gene transcription in response to MCDB in three toxic *Microcystis* strains, one of which was axenic, in order to exclude the interference of other bacteria. We show that MCDB induce MC synthesis, and affect the transcriptional response of *mcyD*, a gene encoding a polyketide synthase involved in MC synthesis in *Microcystis aeruginosa*.

1. Materials and methods

1.1. Experimental material

MCDB (TH8) were isolated from Lake Taihu, Jiangsu Province, China and identified as *Sphingomonas* spp. *Pseudomonas aeruginosa* (CCTCC-AB91095) was purchased from Wuhan University (China) as a non-MC-degrading bacterium. PCC7806 was purchased from The Pasteur Culture Collection of Cyanobacteria (PCC, Paris, France). Other strains were from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-Collection, Wuhan, China). The strains were incubated at 25 ± 1°C under illumination of 20 μmol/(m^2 · sec) under a photoperiod of 12 hr.

1.2. Experimental design

The aim of this study was to find out whether MCDB might influence the MC synthetase genes of *Microcystis*. Strains *M. aeruginosa* FACHB905, *Microcystis* sp. FACHB1325, and *M. aeruginosa* PCC7806 were used. Different cyanobacteria cultures were started at the same order of magnitude (10^6 cell/mL) with the same volume (500 mL). The three experiments were conducted in a 1 L Erlenmeyer flasks of BG11 medium under controlled laboratory conditions (25°C and illumination of 25 μmol/(m^2 · sec) on a 12:12 Light:Dark (L:D) cycle). Culture conditions remained the same throughout the study. TH8 and 91095 were inoculated in LB medium every day in order to provide fresh bacteria. An appropriate number of bacteria was centrifuged at 6000 r/min for 5 min. After centrifugation the supernatant was removed and the bacterial cell pellet was resuspended in 5 mL BG11 medium. The first experiment included four treatments: FACHB905 mixed with 5 mL of TH8 (MCDB, 10^9 cfu/mL); FACHB905 mixed with the same concentration of 91095 (control bacteria (CB), 5 mL); FACHB1325 mixed with 5 mL of TH8 (10^9 cfu/mL); FACHB1325 mixed with 5 mL of 91095 (same concentration as the TH8). The second experiment included four treatments: FACHB905; FACHB905 mixed with 5 mL of TH8 (10^8 cfu mL^{-1}); FACHB905 mixed with 5 mL of TH8 (10^9 cfu/mL); FACHB905 mixed with 5 mL of TH8 (10^{10} cfu/mL). The third experiment included three treatments: PCC7806; PCC7806 mixed with 5 mL of TH8 (10^9 cfu/mL); PCC7806 mixed with 5 mL of 91095 (10^9 cfu/mL). All cultures were shaken by hand three times and the same number of TH8 and 91095 as added on the first day was supplemented every day. Depending on the cell density, 10–20 mL of cultures were taken from the flasks each day and filtered through 0.2 μm pore-size filters (Track-Etched Membranes, Whatman® Nuclepore™). The filters were frozen at −80°C until RNA extraction. *Microcystis* cell densities were estimated using a hemocytometer with a Nikon Eclipse E200 microscope (Nikon, Japan). The numbers of bacteria were determined by the coated plate method count with serial dilution.

1.3. RNA extraction, cDNA synthesis, and qRT-PCR amplification

RNA was extracted from filters using an E.Z.N.A. Plant RNA kit (Omega) according to the manufacturer's protocol with the modification in the first step that each filter was resuspended in a lysis buffer supplied in the kit and was crushed using Lysing matrix E tubes in a Fast Prep Instrument (Fast Prep-24, MP Biomedical, USA). The amount and purity of the extracted RNA were determined using comparison of the optical density at 260 and 280 nm by spectrophotometry (Nanodrop 8000, Thermo Fischer Inc., USA) and agarose gel electrophoresis to evaluate integrity. Samples were then stored at −80°C. After digestion with DNase I (Promega), 2 μg of total RNA was reverse transcribed using a RevertAid first-strand cDNA synthesis kit (Thermo Scientific). Two pairs of specific primers were used to quantify the number of copies of the *mcyD* and 16S ribosomal ribonucleic acid (rRNA) genes, respectively (Table 1). All reactions were completed in a total volume of 20 μL, comprising 0.5 mmol/L of each primer, 0.1 mmol/L Taq probe (Invitrogen, CA, USA), 10 μL Bestar Real-time PCR Master Mix (DBI Bioscience, China), 1 μL BSA (3 mg/mL, Sigma), ddH_2O, and template DNA. The qRT-PCR program for *Microcystis* 16S rRNA and *mcyD* was as follows: 95°C for 2 min, followed by 45 cycles of 95°C for 30 sec and 55°C for 1 min. The messenger RNA (mRNA) copy number was determined using the C_t value. The induction ratio was calculated using the $2^{-\Delta\Delta Ct}$ method according to the handbook for the Bio-Rad Real-time PCR system, where:

$$\Delta\Delta Ct = (Ct_{,\text{target gene}} - Ct_{,16s})_{\text{stress}} - (Ct_{,\text{target gene}} - Ct_{,16s})_{\text{control}}.$$

All assays were performed in triplicate and results reported as means (±standard deviation (SD)). Analyses were conducted with Origin 8.0 (OriginLab, USA).

1.4. MC analysis

Extracellular and intracellular MCs were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (IHB, CAS, China) according to Lei's method (Lei et al., 2004). MC content was expressed as equivalents of MC-LR. In the first and second experiment, 500 μL cultures were used to measure MC content. Samples were centrifuged at 12,000 r/min for 3 min and then the supernatants and sediments were used to test extracellular and intracellular content, respectively. Cells were resuspended in 500 μL double-distilled water (ddH_2O) and then ground with liquid nitrogen for the intracellular MC test. In the third experiment, intracellular MC contents were extracted with methanol 75% (V/V), for which the volumes were 5–20 mL depending on the cell density. For intracellular MC measurement, samples should be diluted several times (500–25,000 times in our experiments) depending on the contents.

1.5. Statistical analysis

Significant differences between control and treated samples were determined using analysis of variance (ANOVA) with R. Differences were considered to be significant at $p < 0.05$.

2. Results

2.1. *Microcystis* growth and *mcyD* expression under the influence of MCDB

Strains FACHB905 and 1325 did not exhibit any differences in growth when two kinds of bacteria (MCDB and *P. aeruginosa* as CB) were respectively added, reaching similar cell numbers during the experiment (Fig. 1a). During the 4 days of culturing, the extracellular MC (EMC) concentration in the cultures decreased markedly in the presence of MCDB, but no changes were observed in the presence of CB (Fig. 1b). In contrast, the intracellular MC (IMC) concentration increased in the presence of MCDB and the IMC concentration remained unchanged with CB (Fig. 1c). EMC and IMC showed opposite trends when MCDB were added, but showed no difference from each other in the presence of CB. Fig. 1d shows a plot of the relative expression of the *mcyD* gene. In strains 905 and 1325, this gene was overexpressed on treatment with MCDB after 2, 3, and 4 days of growth, whereas in treatments with CB no significant changes in the gene expression occurred.

2.2. Different concentrations of MCDB influence *mcyD* gene expression

We also tested FACHB905 growth with different concentrations of MCDB (1:0.1, 1:1, and 1:10 ratios), and there were no significant differences in growth trends (Fig. 2a). The EMC and IMC in the cultures decreased and increased respectively over time after addition of different concentrations of MCDB (Fig. 2b, c). Fig. 2d shows that expression of *mcyD* was up-regulated in response to MCDB stress in the three treatment groups, especially on treatment with the highest MCDB concentration.

Table 1 – Sequences of the primers used in this study for *mcyD* and *16S rRNA*.

DNA target	Primer	Sequence (5′–3′)	Reference
Microcystis 16S rRNA	184F	GCGGCRAGGTGAAAMCTAA	(Neilan et al., 1997)
	431R	AATCCAAARACCTTCCTCCC	
	Probe (Taq)	FAM-AAGAGCTTGCGTCTGATTAGCTAGT-BHQ-1	(Rinta-Kanto et al., 2005)
mcyD	F2	GGTTCGCCTGGTCAAAGTAA	(Kaebernick et al., 2000)
	R2	CCTCGCTAAAGAAGGGTTGA	
	Probe (Taq)	FAM-ATGCTCTAATGCAGCAACGGCAAA-BHQ-1	(Rinta-Kanto et al., 2005)

FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1.
rRNA: ribosomal ribonucleic acid.

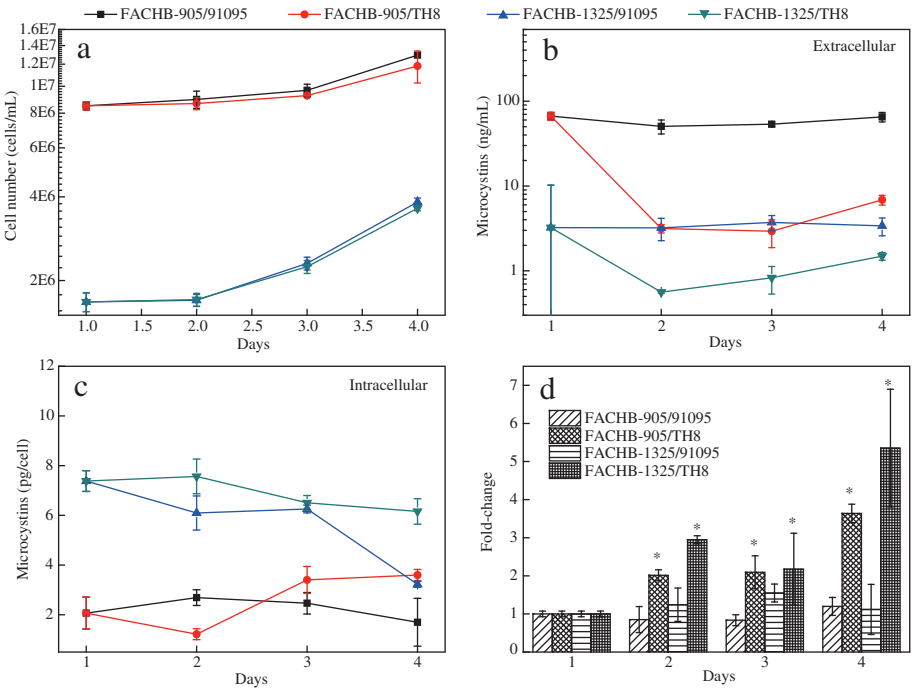

Fig. 1 – Gene expression of unicellular and colony *Microcystis* in the presence of microcystin-degrading bacteria. (a) Changes in cell number with time; (b) changes in extracellular MC concentration; (c) changes in intracellular MC concentration; (d) changes in *mcyD* gene expression with time. Error bars indicate the SD (n = 3), $^{*}p < 0.05$. MC: microcystin; SD: standard deviation.

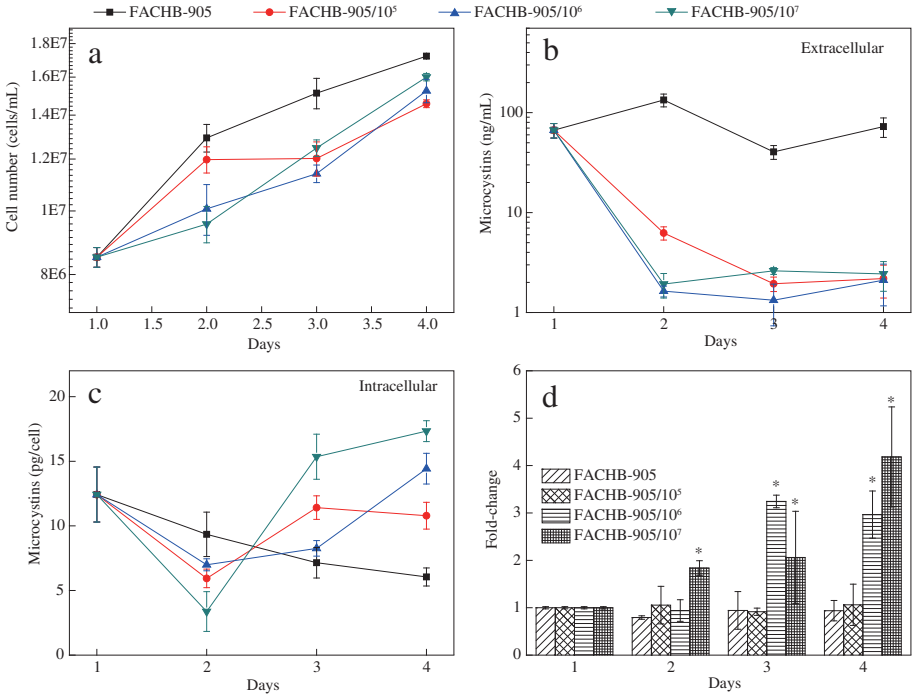

Fig. 2 – Different concentrations of microcystin-degrading bacteria influence FACHB905. (a) Changes in cell number with time; (b) changes in extracellular MC with time; (c) changes in intracellular MC with time; (d) changes in *mcyD* gene expression at different concentrations of MC-degrading bacteria. Error bars indicate the SD (n = 3), $^{*}p < 0.05$. MC: microcystin; SD: standard deviation.

2.3. *mcyD* transcriptional analysis of the MCDB induced response in axenic algae-PCC7806

In order to eliminate the interference of bacteria associated with *Microcystis*, we performed an experiment where bacteria were added into an axenic PCC7806 culture. There were no significant differences in growth between the three treatment groups (PCC7806 alone, PCC7806 + CB, and PCC7806 + MCDB) (Fig. 3a). Compared to controls and CB treatment, expression of *mcyD* following MCDB treatment showed little variation over the first 3 days, but there was an obvious up-regulation from the fourth day to the end of the experiment. Gene expression levels under the influence of MCDB were 21.3 times higher compared with controls at the start of the experiment at the ninth day and 19.8 times at the tenth day (Fig. 3b). During the 10 days of culturing, the IMC concentration in the cultures increased markedly in the presence of MCDB, but no changes were observed in the presence of control and 91095 (CB) (Fig. 3c). Our results showed that *mcyD* gene expression increased when MCDB were added for the second time (on the fourth day), suggesting that MCDB promoted synthesis of MCs in *Microcystis*.

3. Discussion

Our analysis of the variation in *mcyD* gene expression and IMCs and EMCs in response to MCDB by different *Microcystis* strains revealed that MCDB clearly affected *mcyD* transcription and MC production. The findings presented here show increases in MC peptide synthesis and polyketide synthase gene transcription as a result of MCDB.

The TH8 bacteria used in our experiments belongs to the *Sphingomonas* spp. type of MCDB and was isolated from field water (Gan et al., 2012). Previous investigations have suggested that the distinct gene cluster *mlrABCD* of *Sphingomonadaceae* is involved in MC degradation; it encodes an enzymatic ring cleavage system and thus linearizes the MCs (Shimizu et al., 2012). A meta-analysis of data on 16S rRNA and *mlrA* (microcystinase) genes and diversity of isolates known to degrade cyanobacterial toxins revealed that such bacteria belong primarily to the phylum Proteobacteria, including several strains of *Sphingomonas* and two strains belonging to the *Methylobacillus* and *Paucibacter* genera respectively. Other strains belonged to the genera *Arthrobacter*, *Bacillus*, *Ochrobactrum*, and *Lactobacillus* (Kormas and Lymperopoulou, 2013; Kato et al., 2009; Jing et al., 2014). Our results show that added bacteria (either *P. aeruginosa* 91095 or TH8) had no effect on the growth rate of *Microcystis*. Some research combining ecological knowledge on the distribution, abundance, and dynamics of the bacteria associated with toxic cyanobacterial blooms in field water illustrated that different species of bacteria accompany the toxic cyanobacteria and are more diverse when cyanobacterial toxins are present (Briand et al., 2009). However, no evidence had been produced showing that MCDB can influence toxic *Microcystis* abundance during a bloom.

Another interesting result is that *mcyD* gene transcription levels in colony *Microcystis* were higher than in unicellular *Microcystis* after 4 days of MCDB stimulation. The main difference between colony and unicellular *Microcystis* is in the mucilage that always surrounds *Microcystis* (Maruyama et al., 2003). Wu and Song (2008) found that colony *Microcystis* usually had higher levels of EPS and polysaccharide content in the mucilage than unicellular *Microcystis*. During blooms, numerous bacteria are known to be attached to the mucilage (Brunberg, 1999). Mucilage has also been proved to be a habitat for bacteria that exert their specific function to utilize substrate and nutrients from decaying *Microcystis* cells. Maruyama et al. (2003) found that MCDB existed in a limited area of the mucilage of *Microcystis*, so that the concentration dynamics of these bacteria were synchronized with increases in the concentration of cell-bound MC. This suggests that MCDB in the mucilage respond to changes in the concentration of cell-bound MC. These findings can explain our experimental results, in that the IMC and *mcyD* expression levels of colony *Microcystis* were higher than those in unicellular *Microcystis*, because the abundance of MCDB in colony *Microcystis* mucilage was richer than for the unicellular culture. In this experiment we used an axenic strain, PCC7806, in order to compare with FACHB905 and FACHB1325 that were contaminated by associated bacteria in their cultures. The results showed the same *mcyD* gene transcription trend for axenic and non-axenic *Microcystis*.

The most noteworthy change in a *mcyD* transcription level variable that coincided with elevated IMC was the dramatic increase as a result of MCDB. MCs, as secondary metabolites of *Microcystis*, have been extensively studied. Their biological functions can be summarized as: promote colony formation and enhancement of colony (Gan et al., 2012; Kurmayer et al., 2003); defend against herbivorous zooplankton (Rohrlack et al., 2003; Yang et al., 2006; Yang et al., 2008); act as allelochemicals (Leão et al., 2009); and act against light-induced oxidative stress

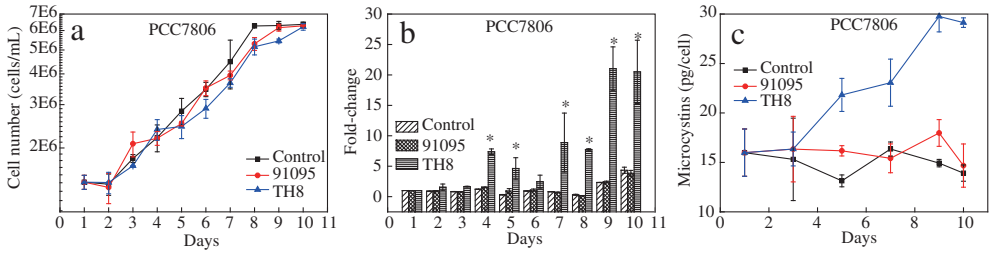

Fig. 3 – Gene expression of axenic *Microcystis* PCC7806 under the influence of MC-degrading bacteria. (a) Changes in cell number with time; (b) changes in *mcyD* gene expression with time; (c) changes in intracellular MC concentration. Error bars indicate the SD ($n = 3$), *$p < 0.05$. MC: microcystin; SD: standard deviation.

(Zilliges et al., 2011; Meissner et al., 2013). From our results, it is possible to infer that the increase in IMC and *mcyD* transcription in *Microcystis* in direct or indirect response to MC consumption could be an immediate response of *Microcystis* as a mechanism of self-defense.

Further studies to elucidate the mechanisms involved in interactions between MCDB and *Microcystis* are now being undertaken. In summary, the results reported here, showing increased *mcyD* transcription and MC production under the influence of MCDB, are in agreement with some recent reports that point to an intracellular function of MC related to protection mechanisms (Penn et al., 2014). Therefore, in our experiments, higher levels of transcript and metabolite were perhaps produced for protection of cells under environmental and biological stress. Further studies may confirm this putative function of MC in the cellular metabolism, to partially elucidate the toxin dynamics observed in field water.

4. Conclusions

Biodegradation by MCDB, an activity of environmental bacteria, appears to be the main pathway that leads to the decrease of MCs in bloom water environments. This study reported an increase in *mcyD* transcription and MC production under MCDB induction and provided molecular evidence that there are interactions between toxic *Microcystis* and MCDB.

Acknowledgments

This work was supported by the Chinese Academy of Sciences (No. KSCX2-EW-Z-3), the National Natural Science Foundation of China (Nos. 31370418, 31123001), and the State Key Laboratory of Freshwater Ecology and Biotechnology (No. 2011FBZ16).

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