The first study on the effects of microcystin-RR on gene expression profiles of antioxidant enzymes and heat shock protein-70 in *Synechocystis* sp. PCC6803

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A R T I C L E   I N F O

A B S T R A C T

Microcystins are heptapeptide toxins produced by cyanobacteria. Microcystin-RR (MC-RR) is a common variant among the 80 variants identified so far. There have been many investigations documenting the toxic effects of microcystins on animals and higher plants, but little is known on the toxic effects of microcystins on algae, especially at molecular level. We studied the effects of MC-RR on gene expression profile of a few antioxidant enzymes and heat shock protein-70 (Hsp70) in *Synechocystis* sp. PCC6803. After two days post-exposure, a high dose toxin (5 mg/l, about 4.8×10^{-3} mM) significantly increased expression levels of the genes *gpx1*, *sodB*, *katG*, *acnB*, *γ-TMT* and *dnaK2*, while a relatively low dose toxin (1 mg/l, about 9.63×10^{-4} mM) induced a moderate and slow increase of gene expression. Our results indicate that MC-RR could induce the oxidative stress in *Synechocystis* sp. PCC6803 and the increase in gene expression of antioxidant enzymes and Hsp70 might protect the organism from the oxidative damage. In addition, cell aggregation was observed during the early period of exposure, which might be a specific oxidative stress reaction to MC-RR.

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1. Introduction

The increase of human activities along with the deficiency of water management has led to a massive growth of cyanobacterial blooms in many freshwater bodies all over the world (Paerl et al., 2001). Microcystins (MCs) produced by cyanobacteria have been given special attention for their adverse effects on other organisms. So far, more than 80 microcystin isoforms have been identified (Fastner et al., 2002). The major isoforms are microcystin-LR (MC-LR), microcystin-RR (MC-RR) and microcystin-YR (MC-YR).

Previous studies have focused mainly on the negative effects of MCs on mammals and higher plants. A well-known molecular toxic mechanism of MCs was their irreversible inhibition of protein phosphatases PP1 and PP2A

(MacKintosh et al., 1990). Recent evidences suggested that oxidative stress was also involved in the toxicity on plants and aquatic macrophyte (Pflugmacher, 2004; Yin et al., 2005a,b). In the last few years, the increasing attention has been paid to the toxicity of microcystin on algae. Singh et al. reported that a high dose of MC-LR could inhibit the photosynthetic process of *Nostoc muscorum* and *Anabaena* BT1 after a short exposure (Singh et al., 2001). Hu et al. (2004, 2005) demonstrated that MC-RR (100 μg/l) could induce the physiological and biochemical alteration as well as the antioxidative response in *Synechococcus elongatus*. Moreover, oxidative stress was observed in *Synechocystis* sp. PCC6803 cells treated with lower concentration of MC-LR (0.05–1.0 μg/l) (Vassilakaki and Pflugmacher, 2008). Although it has been well documented that MCs were transported into liver via bile acid transport proteins (Eriksson et al., 1990), there was no effective microcystin transport system found in plant or algae cells so far (Suchy, 1993). In *Arabidopsis thaliana* suspension cells, it was

suggested that microcystin might enter cells by a negative diffuse way (Yin et al., 2005a).

In spite of extensive studies of microcystins-mediated deleterious effects, there has been very limited information on the effects of MCs on gene expressions of other organisms. Bulera et al. (2001) observed the up-regulation of detoxification genes in the liver of rats exposed to MC-LR for 3 or 6 h. At the same time, Fu and Xie (2006) reported that MC-LR could induce the transcriptional alteration of nine glutathione S-transferase genes in the liver of common carp. In HepG2 cells, MC-LR elevated the expression of tumour suppressor gene $p53$ and its following downstream-regulated genes (Žegura et al., 2008). Yet, to our knowledge, no study is reported for the changes of gene expressions in plant or alga cells after exposure to MCs.

Synechocystis sp. PCC6803 is an ideal model organism to study the molecular toxicology of MC-RR partially because it grows very fast and performs a plant-like oxygenic photosynthesis. Most importantly, its genome has also been completely sequenced. In the present study, PCC6803 was used to further characterize the mechanism of MC-RR induced toxicity. By using quantitative real-time PCR, we analyzed the expression pattern of $dnaK2$ gene (encoded for Hsp70) and several antioxidant related genes, including $sodB$ (encoded for FeSOD), $gpx1$ (encoded for glutathione peroxidase), γ-TMT (encoded for gamma-tocopherol methyltransferase), $katG$ (encoded for catalase peroxidase), and $acnB$ (encoded for aconitate hydratase). Further, we found that a chronic exposure to MC-RR could affect PCC6803 growth.

2. Materials and methods

2.1. Strain and growth conditions

The axenic strain Synechocystis sp. PCC6803 was obtained from Dr. X. Xu of Institute of Hydrobiology, Chinese Academy of Sciences. The organism was cultured photoautotrophically in BG11 medium in 250-ml flasks at 28–30 °C under fluorescent lamps (50 $\mu mol/m^2/s$).

2.2. Toxin

MC-RR was isolated and purified from surface blooms (mainly Microcystis aeruginosa) collected from Lake Dianchi in China using an improved Ramanan method. In this method, the extraction of Microcystis cells was sequentially applied to an octadecylsilyl (ODS) cartridge and semi-prep-LC (Waters 600, USA). The content of purified MC-RR was over 99% and its identity was confirmed by LC–MS^n using the ion trap (Thermo Electron Corporation, San Jose, CA, USA). For MC-RR, precursor ion was $[M+2H]^{2+}$ at m/z 520.0. Collision energy was 37%. Product ions (m/z) included 904.4, 887.4, 754.3, 596.2, 505.9, 452.9, 440.1, 298.4 and 285.1, which are identical with that of the standard sample (MC-RR, Wako Pure Chemical Industries, Japan).

2.3. MC-RR exposure

MC-RR dissolved in distilled water was filter sterilized and added to the liquid BG11 medium to a nominal concentration of 0, 1 (9.63 × 10^{-3} mM) and 5 mg/l (4.8 × 10^{-3} mM), respectively. Growth of Synechocystis cultures was monitored by recording the optical density at 730 nm (OD_{730}) with a Shimadzu UV-1601 spectrophotometer. Cells in late-logarithmic growth phase were used as inoculation for new batch cultures and exposed to MC-RR in 200-ml volumes in sterilized flasks. The initial cell density of the replicates was set to approximately 0.07 OD_{730}. The experiment was performed three times and every treatment was done on three parallel flasks.

2.4. Concentration determination of MC-RR in the medium

At the time points of Days 0 (immediately after exposure of Synechocystis cells to MC-RR), 2, 4, 6, 8, 10 and 12, 4-ml culture cells were sampled, respectively. The samples were centrifuged at 12 000g for 10 min, and the supernatant was used to determine the concentration of MC-RR. MCs samples were prepared according to the method of Park and Lwami (1998) with minor modifications. 1-ml supernatant of the samples was applied to a C18 reversed phase cartridge (0.5 g), which had been preconditioned by washing with 10-ml 100% methanol and 10-ml distilled water. The column was washed with 10-ml distilled water. Elution from the column with 10-ml 90% methanol yielded the MC-containing fraction and the MC-containing fraction was evaporated to dryness. The MC-containing fractions were finally dissolved in distilled water, and used for detection and identification of MCs by liquid chromatography–mass spectrometry (Finnigan LC–MS system) according to Zhang et al. (2007) with minor modifications.

Recovery experiments were performed in sixplicate spiked water as described previously (Park and Lwami, 1998): 1 ml 1 $\mu g/ml$ of purified MC-RR was applied to 0.5 g C18 reversed phase cartridge, and the column was washed and eluted according to the above method of MC-RR samples preparation. The recovery of the analytical method was calculated.

2.5. Growth measurements

Cells were exposed to MC-RR on Day 0. The growth was firstly monitored on Day 2 and then Days 4, 6, 8, 10 and 12 by measuring OD_{730}. The cultures were vortexed thoroughly to avoid possible effect of cells aggregate before measuring each time. Growth curves based on the changes in OD_{730} were constructed.

2.6. Total RNA extraction, reverse transcription

At all time points listed above, suitable culture cells were harvested from samples treated with or with no MC-RR by centrifugation at 6000g for 5 min. The pellets were preserved in liquid nitrogen for total RNA extraction. Total RNA was isolated using Trizol reagent (Invitrogen) and then treated by DNaseI to remove the contaminant DNA. The purified RNA (1 μg) was reversely transcribed with random hexamer primer using first strand cDNA synthesis kit (Toyobo, Japan). The resultant cDNA was then diluted 10-fold and kept at −20 °C for real-time PCR.

2.7. Quantitative real-time PCR (Q-PCR)

The gene sequences were obtained from Genome Database for Cyanobacteria (CyanoBase, http://www.kazusa.or.jp/cyano/cyano.html). All the primers used in Q-PCR were listed in Table 1. The specification of each pair of primers was confirmed by randomly sequencing three clones of PCR products, and further confirmed by the melting curve analysis using Q-PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid. Only primers with similar amplification efficiency were used in this experiment. 16S rRNA gene was used as the internal control. Q-PCR was conducted by amplifying 1.0 μl of 10-fold diluted cDNA with the SYBR Green qPCR kit (Finnzymes Company, Finland) on a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA). Reaction mixtures (20 μl) were pre-incubated 5 min at 95 °C, and then amplified by 40 cycles of 94 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that a single PCR product was detected. PCRs without addition of the template were used as blank controls. After completion of the PCR amplification, data were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, MA). The experiments were repeated twice with three replicates.

2.8. Statistics

All values were expressed as mean ± standard deviation. One-way ANOVA was used to elucidate if there were significant differences between treatment groups and the control groups ($p < 0.05$ was considered as a statistically significant difference).

3. Results

3.1. The kinetics of MC-RR concentration changes in medium

Although the initial nominal concentrations of MC-RR in exposure medium were 1 (9.63×10^{-4} mM) and 5 mg/l (4.8×10^{-3} mM), the real concentrations were approximately 0.73 mg/l (7.03×10^{-4} mM) and 3.75 mg/l (3.6×10^{-3} mM), respectively. The concentrations of MC-RR in the two treated groups had a trend of decline during the whole experiment period. However, the decrease was very slight when compared with the initial nominal concentration (Fig. 1). The average recoveries ($n = 6$) of MC-RR in water were 76.29% (range, 74.58–78.74%).

3.2. Growth inhibition

Compared with the control, MC-RR-exposed cultures showed a slight growth inhibition at two doses. But this inhibitory effect was not significant (Fig. 2).

3.3. MC-RR induced cell aggregation of PCC6803

After 1 d exposure to MC-RR at two concentrations, distinct aggregation of PCC6803 cells was observed (Fig. 3). MC-RR-exposed cells tended to form large aggregates, which were often suspended in the medium or floated on the surface, whereas cells in the control group were usually evenly settled down at the bottom of the flasks. The aggregation of cells was intensively evident at 2 d of exposure to 5 mg/l of MC-RR. However, this phenomenon became less evident when cell density reached higher level, usually after 6 d of exposure to MC-RR.

3.4. Gene expression profile

By using quantitative real-time PCR, gene-specific transcription was quantified in PCC6803 cells exposed to two doses of MC-RR for 2, 4, 6, 8, 10 and 12 d (Fig. 4).

The expression of gpx1 was significantly up-regulated to about 12-fold of control level within 2 d post-exposure to 5 mg/l of toxin. However, from 4 d to 12 d, its expression was significantly suppressed compared with the controls. While at the low dose, it began to rise after 4 d. On the 6th day, it reached the highest level but was only about 2-fold of control and then began to decrease. The expression profile of acnB was similar to that of gpx1. There was a significant up-regulation on the 2 d post-exposure to the 5 mg/l of MC-RR. However, at the 1 mg/l group, the expression of acnB showed no significant alteration except for a slight up-regulation at 2 and 6 d points.

Table 1
Primer sequences used for Q-PCR analyses.

| Gene (*Synechocystis* identifier)		Primer sequences (5′–3′)	Size (bp)
sodB (slr1516)	F	GCTGGGCTTGGTTGGTGTTAGAC	203
	R	AGCCGCCACAAAGTCCCAGTT	
gpx1 (slr1171)	F	CTGCCAATGCCTTGGATGGTTC	125
	R	GTATAGAGCCTGGAGTCCTTGGT	
γ-TMT (slr0089)	F	CTACGACGACTCCAGCGGTCT	142
	R	CACTGCCCAGGCCAATAGTTCT	
16sRNA (rrn16Sa)	F	GATGAACGCTGGCGGTATGCCT	142
	R	GGCACATTGGGTATTAGCAGTCGT	
katG (sll1987)	F	CATCGCTACGGCAGTGAAGACC	178
	R	GGGCAACGGTTTCCTCGTCAT	
acnB (slr0665)	F	GCCAAGGGGCGGTGGACTTAT	187
	R	TTGGCGTAGCCGTTGGTATCACT	
dnaK2 (sll0170)	F	GCACGACCCCATCGGTGGTT	170
	R	GCTGTAGGCAACTTCGGTGGCT	
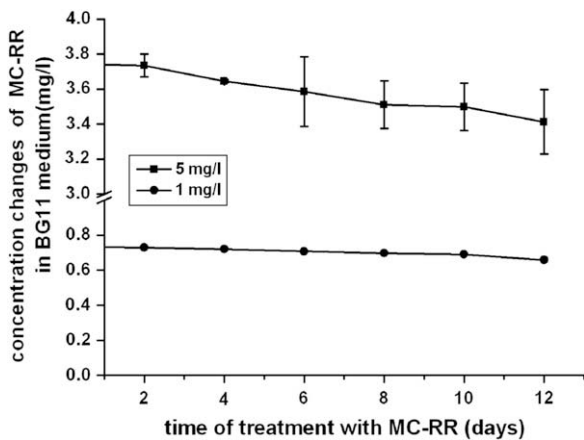
Fig. 1. Changes of MC-RR concentrations in medium exposed to 1 or 5 mg/l of MC-RR. Each point represents mean $\pm$ SE.

The expression of *sodB*, γ-*TMT* and *dnaK2* was significantly up-regulated compared with the controls after 2 d exposure to 5 mg/l of MC-RR. With further exposure for 4, 6 and 8 d, their expressions declined progressively to the control level. However, after 10 d post-exposure, these genes were up-regulated again. When exposed to low dose, the expression of *sodB* and γ-*TMT* reached a peak level at 6 d. From then, their expression began to decrease. For *dnaK2*, however, its expression was not significantly modulated after exposure to 1 mg/l of MC-RR.

As shown in Fig. 4, the expression of *katG* gene showed fluctuation. It was significantly up-regulated at 2 d post-exposure to the 5 mg/l of MC-RR, whereas evidently down-regulated at 4, 8 and 12 d post-exposure. At the low dose group, the expression was up-regulated slightly at 2 and 8 d but down-regulated at other time points.

4. Discussion

It is well known that SOD converts the superoxide anion radicals to hydrogen peroxide. Previous studies have demonstrated that MCs induce formation of reactive
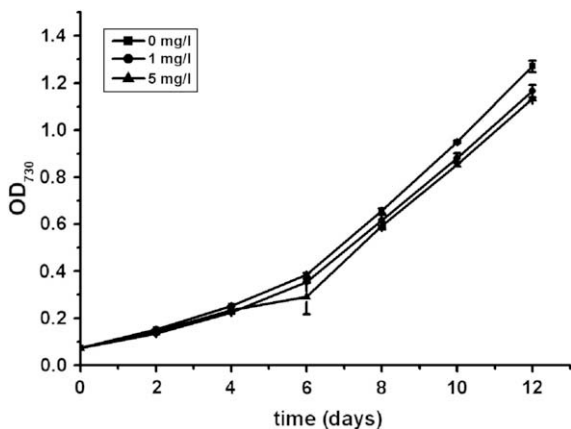

Fig. 2. Growth responses of *Synechocystis* sp. PCC6803 in the presence of 0, 1 and 5 mg/l of MC-RR. Each point represents mean $\pm$ SE. OD, optical density.

oxygen species (ROS), such as superoxide radicals and hydrogen peroxide (Ding et al., 2000; Hu et al., 2004). *sodB*, which encodes FeSOD, is the only *sod* gene in PCC6803 (Ushimaru et al., 2002). In this study, the significant up-regulation of *sodB* suggested a cell response against oxidative stress-induced MC-RR in PCC6806. Vassilakaki and Pflugmacher (2008) reported an elevated SOD activity in PCC6803 exposed to both MC-LR and cell-free crude extract, which was in line with our results. In addition, *sodB* gene could be up-regulated by various other environmental stresses, such as high and low temperature, high salinity and light (Ushimaru et al., 2002).

In the present study, a significant increase in *gpx1* mRNA was also detected. Interestingly, the expression profile of *gpx1* was very similar to that of *sodB*. The up-regulation of *gpx1* gene probably protected the cells from destructive effects of H_2O_2 resulting from the increased *sodB*. It has been reported that MC-LR could increase the transcription of *gpx* in liver of mice and tilapia (Gehringer et al., 2004; Wang et al., 2006). In *Synechocystis* PCC6803, *slr1992*, which encoded another glutathione peroxidase, was also up-regulated by high light (Hihara et al., 2001). Meanwhile, elevated glutathione peroxidase activity was detected in *Synechocystis* PCC6803 cells exposed to MC-LR (Vassilakaki and Pflugmacher, 2008).

Besides *gpx1*, PCC6803 had a unique type of H_2O_2-detoxifying enzyme, catalase/peroxidase, which was encoded by *katG* gene (Jakopitsch et al., 1999). Although it was reported that *katG* could not be induced by external H_2O_2 treatment or other environmental stresses in PCC6803 (Ushimaru et al., 2002), the obvious up-regulation of *katG* in PCC6803 cells exposed to MC-RR was observed in the present study. Our result might indicate the important role of catalase/peroxidase in scavenging internal H_2O_2 resulting from the increase of *sodB*. In addition, the three genes of *sodB*, *gpx1* and *katG* had a similar expression pattern. Therefore, we suggested that the coordinated expression of antioxidant enzyme genes was essential for an effective defense against ROS induced by MC-RR in PCC6803.

Aconitate hydratase is a multifunctional enzyme. Apart from its role in respiration, aconitate hydratase also served as sensor of ROS (Rouault and Klausner, 1996). The up-regulation of *acnB* gene in PCC6803 might reflect the level of oxidative stress induced by MC-RR. Thereby, the whole antioxidant system might be activated by the modulation of *acnB* gene, so as to avoid the more seriously oxidative damage. This assumption was supported by the expression profile of the aforementioned antioxidant enzymes.

In addition to various antioxidant enzymes, there were convincing data showing the ability of induced Hsp to protect against oxidative stress (Arrigo, 1998; Gautier et al., 2001). In the present study, the significant up-regulation of *dnaK2* (which encoded for Hsp70, the main inducible Hsp) was observed in PCC6803 cells when exposed to 5 mg/l of MC-RR. Our results were in accordance with that of Jayaraj et al. (2006), who reported a time-dependent increase of this gene over control at 1 LD_{50} dose exposure to MC-LR in mice. The up-regulation of *dnaK2* might be attributed to the oxidative effect of MC-RR, which enhanced the expression of Hsp70 as a cellular defense. The mechanism for this
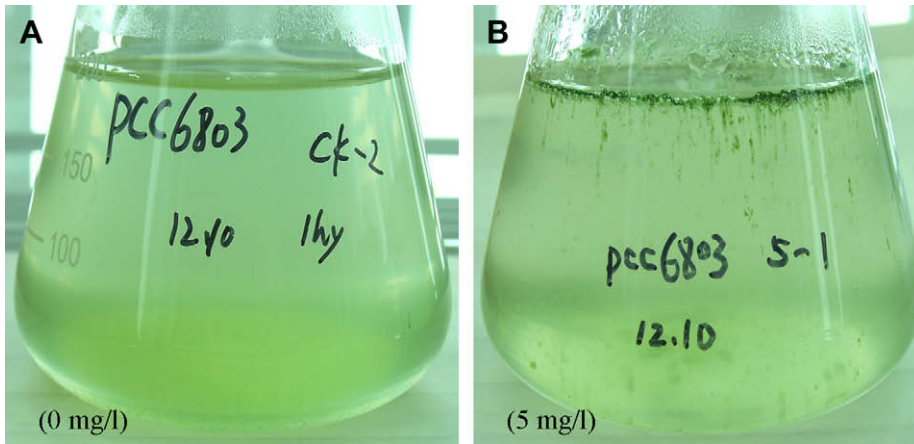
Fig. 3. (A) Control group of the strain. Cells were randomly dispersed in the flask at 4 d. (B) Strains treated with 5 mg/l of MC-RR after 4 d post-exposure. Cells were aggregated together and concentrated mostly in the surface of the medium.
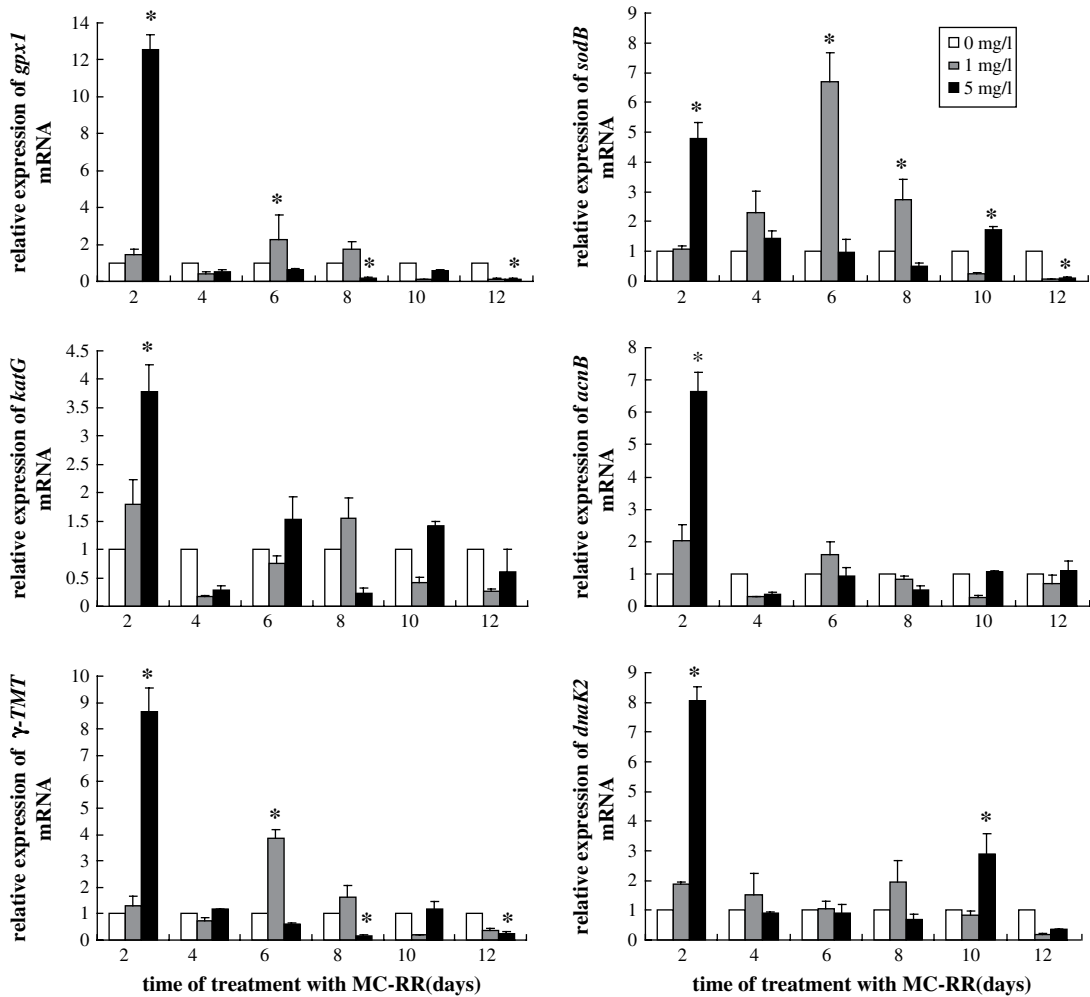

Fig. 4. The temporal changes of gene transcripts in PCC6803 cells exposed to MC-RR. Black columns represent 5 mg/l group, gray columns represent 1 mg/l group, and white columns represent control group. (* indicates significant change at $p < 0.05$.)

protection was thought to be due to the ability of Hsp to decrease ROS by increasing the level of GSH (Mehlen et al., 1996); preventing the malfolding of antioxidant enzymes and enhancing their levels and activities (Halliwell, 1987; Currie and Tanguay, 1991). In addition, Hsp could also protect against oxidative stress-induced apoptosis by functioning at multiple sites in the apoptotic-signalling pathway (Corrido et al., 2001).

α-Tocopherol (vitamin E), a lipid-soluble organic molecule synthesized only by oxygen-evolving phototrophs (Collins and Jones, 1981), could deactivate ROS, and prevent the propagation of lipid peroxidation by scavenging lipid peroxyl radicals (Munné-Bosch, 2005). It was reported that tocopherol-deficient mutants of Synechocystis PCC6803 grew poorly when challenged with oxidative stress (Maeda et al., 2005). γ-TMT gene encodes γ-tocopherol methyltransferase, which catalyzes the methylation of γ-tocopherol to α-tocopherol during the synthesis of α-tocopherol. In this study, MC-RR could up-regulate the expression of γ-TMT gene. Therefore, it would inevitably result in the elevation of α-tocopherol, and contribute to the inhibition of lipid peroxidation and the avoidance of oxidative damage induced by MC-RR.

In addition to the changes in gene expression, we also observed the aggregation of PCC6803 cells after exposure to MC-RR. The aggregation was very obvious during the early period, when the genes of antioxidant enzymes were up-regulated significantly. Therefore, the aggregation of cells might reflect a specific stress reaction to the oxidative stress in PCC6803. Similar phenomenon was also observed in unicellular M. aeruginosa and Scenedesmus quadricauda in the presence of MCs (Sedmak and Elersek, 2005). The aggregation of M. aeruginosa cells was inferred beneficially to a denser formation of cyanobacterial blooms (Kurmayer et al., 2003; Sedmak and Elersek, 2005). However, the aggregation of PCC6803 cells in this study obviously had a different significance.

The real concentrations of MC-RR were lower than the initial nominal concentrations. There were several possible reasons for this difference: Firstly, the MC-RR recovery (average recoveries of MC-RR in water were 76.29%) was the main reason. Secondly, some MC-RR might have been taken up by the Synechocystis cells or adhered to the surface of the cells and seston. It is also possible that some MC-RR were decomposed during the process of the experiment. However, no obvious degradation of MC-RR occurred during the whole experiment period. Therefore, the effect of degradation on the expression profiles of genes as well as the aggregation of cells might be ignored. In this study, the toxin concentration used was relatively high compared with that in natural blooming waters. However, it has been indicated that MCs concentration of up to 1.8 mg/l can occur for short periods following the lysis of cyanobacterial scum (Jones and Orr, 1994).

In conclusion, MC-RR could induce the oxidative stress in Synechocystis sp. PCC6803 and the increase in gene expressions of antioxidant enzymes and Hsp70 might protect the algae from the oxidative damage. At the same time, it was the whole set of antioxidant defenses rather than a single antioxidant that helped PCC6803 cells to withstand MC-RR induced stress.

Conflict of interest

The authors declare that there are no conflicts of interest.

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