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# Growth and Antioxidant System of *Escherichia coli* in Response to Microcystin-RR

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**Abstract** Microcystins are a kind of cyclic hepatoxins produced by many species of cyanobacteria. The toxic effects of microcystins on animals and plants have been well studied. However, the reports about the effects of microcystins on microbial cells are very limited. In present paper, *Escherichia coli* was undertaken to determine the effect of microcystin-RR. These results suggested that microcystin-RR could prolong the growth of *E. coli* when exposed to high concentrations of microcystin-RR and cause the accumulation of ROS and induce the oxidant stress for a short time. The antioxidant system protects *E. coli* from oxidative damage.

**Keywords** Microcystin-RR · *Escherichia coli* · Growth · Antioxidant system

Blooms of cyanobacteria have occurred in many regions all over the world and produce a number of toxins, including dermatotoxins, neurotoxins, and hepatotoxins. Among hepatotoxins, microcystins are the most widespread distributed and are found to have more than 60 kinds of structural analogs (Carmichael 1994). The toxic effects of microcystins on animals and plants have been widely carried out. It was reported that microcystins could result in the death of invertebrate, fish, birds and other wild and domestic animals, and bioaccumulation of microcystins by aquatic organism including fish, shellfish, and zooplankton determines the risk of health problems among animal and people that eat food contaminated with cyanotoxins. Microcystins could also result in the response of antioxidant systems in the different tissues of fish, such as isolated hepatocytes of *Cyprinus carpio* (Li et al. 2003), liver, kidney and gills of *Oreochromis* sp. (Jos et al. 2005) and so on. In plants, it was reported that microcystins could also inhibit the growth and induce the antioxidant system reaction of plant. Oxidative stress may also play an important role in the toxicity of microcystins on plants (Yin et al. 2005). However, few studies have been tried to elucidate the possible effect of microcystins on microbes, which are equally important in aquatic ecosystem. For the first time, Dixon et al. (2004) reported the permeabilising effects of sub-inhibitory concentrations of microcystin on the growth of *Escherichia coli*. It pointed out that microcystin-RR in combination with cationic detergents and other noxious matters may contribute to modulation of bacterial numbers in some aquatic environments. In our experiment, we used microcystin-RR, which was one of the most common isomers of microcystins, to study the biochemical response of *E. coli*.

## Materials and Methods

Microcystin-RR was isolated from a natural bloom of cyanobacteria which occurred in lake Dianchi in the southwest of China and was purified with the improved HPLC method (Harada et al. 1988). *Escherichia coli* HB101 used in this study was kindly provided by Dr Bian Yan, Wuhan University, China. The cells were cultured in nutrient broth liquid medium and maintained at 30°C in

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darkness. For microcystin-RR toxicity studies, the cells were grown to an $OD_{600}$ of about 0.1 in nutrient broth liquid medium and microcystin-RR was added into the medium to yield a final concentration of 1, 5, 10, and 15 μg/mL. The control group contained 0 μg/mL microcystin-RR. Control and toxin-treated cells were harvested for biochemical measurements after culturing for 0.5, 1, 1.5, 2, 3, 4, 5 h. The effect of microcystin-RR on the growth of *E. coli* was measured by $OD_{600}$ once an hour for 5 h.

For the biochemical measurements, the control and the microcystin-RR treated cultures were harvested by centrifugation at 4°C and 8,000 rpm for 10 min and washed in sodium phosphate buffer (pH 7.8), centrifuged as before again ,and the pellets were resuspended in the same buffer. Cells were disrupted by sonication in a 5-s burst for a total of 6 min with a 6-s cooling period after each burst using an ultrasonic disintegrator below 4°C. Cellular debris was removed by centrifugation for 10 min at 8,000 rpm, the supernatant was used to analyze the content of ROS and the enzyme activities of SOD, CAT, and GR.

The level of ROS was measured by a colorimetric method based on the Fenton reaction. ROS kit was purchased from the Nanjing Bioengineering Institue, China. SOD activity was measured by the inhibition of nitro blue tetrazolium reduction at 560 nm (Beyer and Fridovich 1987). One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the rate of nitro blue tetrazolium reduction under the assay conditions. CAT activity was measured in terms of the decomposition of hydrogen peroxide, which was monitored directly by the decrease in absorbance at 240 nm (Beers and Aizer 1952). With some modifications, the reaction mixture of 3 mL contained 50 mM sodium phosphate buffer (pH 7.0) 1.8 mL, 0.2% $H_2O_2$ 1 mL and the enzyme extract 0.2 mL. GR activity was measured by following the consumption of NADPH in a reaction medium containing 50 mM K-phosphate buffer (pH 7.5), 0.5 mM EDTA, 1.0 mM oxidized glutathione, 0.25 mM NADPH, and 20 μL supernatant in a final volume of 1.5 mL (Lushchak et al. 2005).

The lipid peroxidation products of thiobarbituric acid reactive substances (TBARS) in *E. coli* were measured according to Steels (1994). With some modifications, cells were harvested and resuspended in 5% trichloroacetic acid (TCA), disrupted by sonication in a 5-s burst for a total of 6 min with a 6-s cooling period after each burst using an ultrasonic disintegrator below 4°C. Cellular debris was removed by centrifugation at 4°C for 10 min at 8,000 rpm, the supernatant was used to analyze the content of TBARS. The reaction mixture in a total volume of 3 mL containing 1.5 mL of extracts, 1.5 mL 0.67% thiobarbituric acid, was heated in boiling water for 60 min and then quickly cooled. The absorbance of the supernatant was measured at 532 nm after the mixture was centrifugated at 5,000 rpm for 15 min. TBARS content was determined by subtracting the absorbance value measured at 600 nm.

GSH content was assayed according to the method of Griffith (1980). Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method, using bovine serum albumin as the standard. All experiments were repeated three times. The results were expressed as means ± S.E. All data were evaluated by one-way ANOVA analysis (SPSS 12.0 for windows) with a significant level of $p < 0.05$.

## Results and Discussion

The results in the present study showed that the growth of *E. coli* was significantly prolonged when exposed to microcystin-RR of 10 μg/mL or above, compared with the control. But the $OD_{600}$ only showed a slight decrease when *E. coli* exposed to microcystin-RR of 5 μg/mL or below for 1 h (Fig. 1). The $OD_{600}$ of the high concentration (10 μg/mL or above) toxin- treated group was lower than that of the control during all the experiment. Since toxin-treated *E. coli* cells had to consume a lot of endogenous compounds to conjugate or transform this xenobiotic, the growth and reproduction of *E. coli* was unavoidable to be prolonged. But the dose of microcystin-RR did not have a lethal effect. *E. coli* only showed growth inhibition at the initial growth phase when cells were treated with microcystin-RR. Indeed, gradually the normal rate of growth was re-established and the curves of growth of treated and untreated bacteria became parallel. Similar patterns were also observed in the cyanobacterium *Synechococcus* elongates when exposed to microcystin-RR. Microcysitn-RR made the algal cells had a prolonged

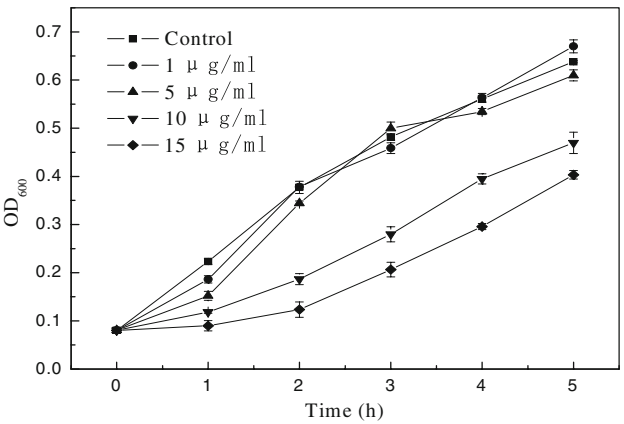


**Fig. 1** Growth response of *E. coli* in the presence of 1, 5, 10, and 15 μg/mL microcystin-RR. $OD_{600}$ of the 10 μg/mL or above toxin-treated group was significantly different from the control ($p < 0.05$). Each point represents the mean of $OD_{600}$ ± SE (OD) optical density

lag growth phase compared with the control (Hu et al. 2005).

In previous studies, it has been found that microcystins could induce oxidative stress in animals and plants as described in the introduction section. In our study, six parameters of ROS, TBARS, GSH, SOD, CAT, and GR are used to evaluate the antioxidant defense of *E. coli* when exposed to microcystin-RR. These parameters are widely used as biomarkers in oxidative stress of bacteria and yeast. Our experiments showed an obvious increase of ROS content in *E. coli* when exposed to microcystin-RR of 10 μg/mL or above for 1 h compared with the control ($p < 0.05$). While it only showed a slight increase of ROS content when *E. coli* was treated with microcystin-RR of 5 μg/mL or below during all the experiment when compared with the control. The increment of ROS in the high concentration of 10 or above toxin-treated group was almost two times higher than that in the control (Fig. 2a). With the increment of exposure time, ROS content of the treatment group decreased gradually while that of the control group constantly kept at a low level over the experiment period. After 5 h exposure, the toxin-treated group had low levels of ROS, which was not nearly different from the control. The increase of ROS suggested that *E. coli* cells were under oxidative stress as the result of exposure to microcystin-RR, which was similar to that of hepatocytes of *cyprinus carpio* (Li et al. 2003) and tobacco BY-2 cells ROS accumulation induced by 50 μg/mL mcirocystin-RR (Yin et al. 2005). After 5 h exposure, the ROS content in the treatment group decreased to the same low levels as that in the control. It indicated that *E. coli* might have eliminated redundant ROS by means of the increment of cell numbers or the increment of antioxidant in *E. coli* cells.

TBARS content indicated lipid peroxidation, which is another manifestation of oxidative damage induced by

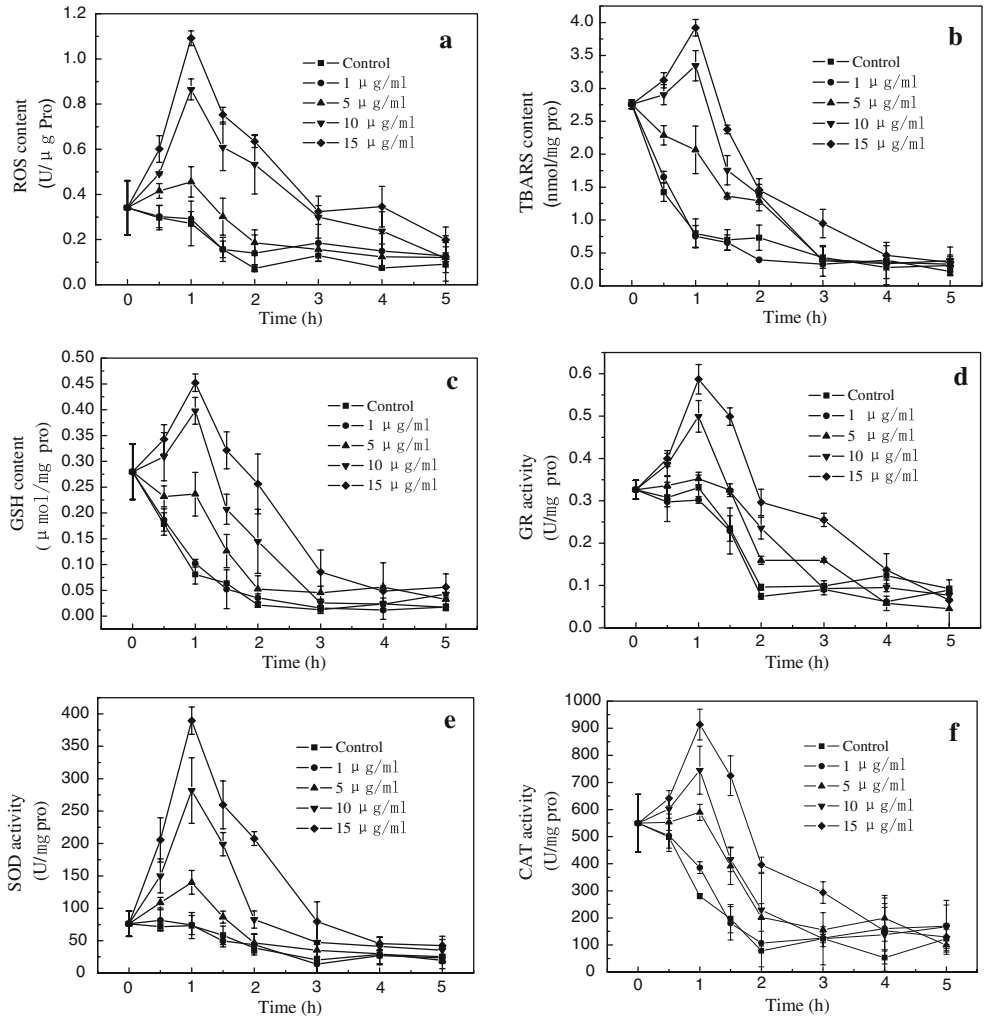


**Fig. 2** Effect of 1, 5, 10, and 15 μg/mL microcystin-RR on the ROS content (**a**), TBARS content (**b**), GSH content (**c**), GR activity (**d**), SOD activity (**e**) and CAT activity (**f**) in *E. coli*. The values in the 10 μg/mL or above toxin-treated group were significantly different from the control after 1 h exposure ($p < 0.05$). Each point represents mean ± SE

abiotic stresses, such as toxin, salinity, extreme temperatures, UV light, etc. cause oxidative stress to bacterial cells either directly or indirectly. Our study showed that the content of TBARS was obviously increased in *E. coli* when exposed to microcystin-RR of 10 μg/mL or above for 1 h compared with that of the control ($p < 0.05$). The treatment group of 1 μg/mL microcystin-RR showed nearly no difference compared with the control in TBARS content. When exposed to 5 μg/mL microcystin-RR for 1 h, the TBARS content was showed a little increase, but it still showed no significant difference from that of the control. Both the TBARS contents of the treatment group and the control group were relatively high at the beginning of the experiment, which may be attributed to oxidative stress caused by the new medium. After *E. coli* was treated with microcystin-RR for 4 h, TBARS content in all the treatment groups reduced to the same level as that in the control. It indicated that oxidative stress had been eliminated in *E. coli* (Fig. 2b).

GSH is one of the most major antioxidant molecules of cells and is considered to play an important role in buffering the cells against stressed environment. It has demonstrated the role of glutathione in the protection of *E. coli* against oxidative stress. It also plays an important role on ROS exclusion, microcystins elimination and detoxification. In our experiment, GSH content of the treatment group was more significantly increased than that of the control when *E. coli* was exposed to microcystin-RR of 10 μg/mL or above for 1 h ($p < 0.05$), while it showed little difference from that of the control when treated with microcystin-RR of 5 μg/mL or below for 1 h. Subsequently, GSH content in the toxin-treated group gradually reduced to the same low level as the control after 3 h (Fig. 2c). It is contrary to some reports of plants and animals (Li et al. 2003; Yin et al. 2005). When plants or animals cells were exposed to microcystin-RR, GSH content decreased for its role in ROS and microcystins elimination. However, GSH content of the treatment group in *E. coli* increased more obviously than that of the control. It was assumed that GSH content in *E. coli* was comparatively low. When it was exposed to microcystin-RR, amounts of GSH were synthesized to clear the toxin, which might stand for the reason that GSH content significantly increased when *E. coli* was exposed to microcystin-RR for 1 h. On the other hand, we determined the activity of GR in *E. coli*. GR was considered to reduce GSSG to GSH and prevent the oxidative damage of cells. In our present work, an obvious increase of GR activity was found when *E. coli* was exposed to microcystin-RR of 10 μg/mL or above for 1 h which was similar to that of GSH (Fig. 2d). It just explained the cause of the increase of GSH contents when *E. coli* was treated with microcystin-RR. While GR activity of the low concentration (5 μg/mL or below) toxin-treated group showed no significant difference from that of the control.

SOD and CAT were found in almost all organisms and are known as important antioxidant enzymes. It was reported that lower concentration of microcystin (0.01 μg/mL) could result in the significant increase of SOD and CAT activity in hepatocytes of common carp (Li et al. 2003). And higher concentration of microcystin (50 μg/mL) also resulted in the obvious increase of SOD and CAT activities in tobacco BY-2 cells (Yin et al. 2005). Our results showed that SOD activity of toxin-treated *E. coli* was significantly increased when exposed to microcystin-RR of 5 μg/mL or above for 1 h compared with the control ($p < 0.05$) (Fig. 2e), and it even reached four times higher than that in the control when exposed to 10 μg/mL or above. It indicated that SOD might play an important role in scavenging ROS. However, when the toxin treatment prolonged, SOD activities of the treatment group decreased, and it had almost no difference from the control after 3 h exposure. The decrease of SOD activities may attribute to the increase of cell numbers and the clearance of ROS. CAT activity of 5, 10 or 15 μg/mL toxin-treated *E. coli* was also significantly increased after 1 h exposure, which is similar to that of SOD (Fig. 2f). With the increment of toxin-treat time, there was almost no difference between the treatment group and the control in *E. coli*. Both SOD and CAT activities increased, which indicated that SOD and CAT were involved in the defense against the stress caused by microcystin-RR. But meanwhile in our experiment, we found that the activities of SOD and CAT showed almost little difference from that of the control when *E. coli* exposed to 1 μg/mL microcystin.

So far as we know, this is the first report that microcystin-RR can prolong the growth and induce oxidative stress in *E. coli*. But it was a transient effect, which peaks at 1 h and then declined and that there was an induction of GSH, SOD, CAT, GR which may be responsible for the decline of ROS with re-acquisition of growth. In our experiment, the toxin concentration we used was comparatively higher than the concentration found in natural environment. However, it has been reported that the concentration of microcystins can reach 1.8 μg/mL for short periods following the lysis of cyanobacterial cells (Jones and Orr 1994), and the rich culture medium promote the growth of *E. coli* to resist the oxidative stress which was manifested by the decrease of ROS content and antioxidant enzymes activities with the toxin-treat time prolonged. Microcystin-RR has no lethal effect on *E. coli*, which was consistent with previous reports that microcystin has been shown previously to have little or no antibacterial activity. In summary, the stress effect of microcystin-RR can induce the accumulation of ROS in *E. coli* for a short period. On the other hand, it could use

the increase of antioxidant enzyme activities to scavenge the ROS, which could prevent the cells from being damaged.

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