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

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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*.

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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(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.