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Frontier Article

Antioxidant responses and degradation of two antibiotic contaminants in *Microcystis aeruginosa*

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ABSTRACT

Cyanobacteria may interact with antibiotic contaminants in aquatic environments, but the interaction effects and mechanisms remain unclear. In the present study, aqueous culture of *Microcystis aeruginosa* was exposed to 50 ng/l–1 μg/l of spiramycin and amoxicillin for seven days. The influences of antibiotics on the antioxidant system of *M. aeruginosa* and the degradation of antibiotics by *M. aeruginosa* were investigated. The activities of superoxide dismutase (SOD) in spiramycin-treated *M. aeruginosa* were stimulated by up to 2.2 folds, while the activities of peroxidase (POD) and catalase (CAT) were inhibited by spiramycin at test concentrations of 500 ng/l–1 μg/l, with a decrease of up to 71% and 76% compared to the control, respectively. The activities of SOD, POD and CAT in *M. aeruginosa* were stimulated by amoxicillin during the whole exposure period, with respective increases of up to 60%, 30% and 120% relative to the control. At test concentrations of 500 ng/l–1 μg/l, the higher MDA contents in spiramycin-treated *M. aeruginosa* indicated a higher toxicity of spiramycin than amoxicillin, possibly due to the accumulation of hydrogen peroxide caused by the inhibited activities of POD and CAT under exposure to spiramycin. The increase of glutathione content, the stimulation of glutathione S-transferase activity and the degradation of each antibiotic were observed in *M. aeruginosa* during the 7-day exposure. At the end of exposure, 12.5%–32.9% of spiramycin and 30.5%–33.6% of amoxicillin could be degraded by *M. aeruginosa* from the culture medium, indicating the ability of *M. aeruginosa* to eliminate coexisting contaminants *via* detoxification.

1. Introduction

The frequent outbreak of cyanobacterial blooms has become a ubiquitous phenomenon in freshwater ecosystems, leading to serious water quality problems such as hypoxia and off-flavor in water bodies and release of cyanotoxins from toxic cyanobacterial strains (Graham et al., 2010; Pan et al., 2006; Veldhuis and Wassmann, 2005). Formation of cyanobacterial blooms are normally regulated by various environmental factors, including nitrogen (N), phosphorous (P), N:P ratio, light intensity, trace metals, temperature, and pH (Davis et al., 2009; Jiang et al., 2008; Xu et al., 2011). Recent studies found that many domestic and industrial contaminants could also affect the growth of cyanobacteria and production of cyanotoxins (Perrona and Juneau, 2011; Wang et al., 2007; Zeng et al., 2009).

Antibiotic contaminants are discharged into aquatic environments after application in public health, veterinary medicine and agriculture, as a result of their incomplete metabolism in humans and livestock (Santos et al., 2010; Zuccato et al., 2010). Antibiotics are effective against environmental microorganisms, especially for bacteria (Kümmerer, 2009). Due to the cell structure similar to bacteria (Stanier and Bazine, 1977), cyanobacteria are more sensitive to antibiotic contaminants compared with other algal species (Crane et al., 2006). Several studies investigated the effects of antibiotic contaminants on *Microcystis aeruginosa*, which is one of the most widely distributed toxic cyanobacteria strains (Ando et al., 2007; Stoichev et al., 2011). Antibiotic contaminants were found to affect the growth and photosynthetic efficiency of *M. aeruginosa* at environmentally relevant concentrations (Halling-Sørensen, 2000; Van der Grinten et al., 2010). A recent study by our group demonstrated that two antibiotic contaminants, spiramycin and amoxicillin, could affect the algal growth and the production and release of microcystins in *M. aeruginosa* (Liu et al., 2012). However, the interaction mechanisms between antibiotic contaminants and *M. aeruginosa* remained unclear.

Exogenous contaminants may generate oxidative stress and trigger antioxidant responses in algae, which could further participate in the regulation of algal growth and numerous physiological processes (Mallick and Mohn, 2000; Rutherford and Krieger-Liszkay, 2001). A few researchers focused on the action mechanisms of allelochemicals, heavy metals and industrial contaminants in *M. aeruginosa*, and verified that antioxidant systems played an important role during the interactions between exogenous chemicals and

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M. aeruginosa (Gao and Tam, 2011; Qian et al., 2012b; Shao et al., 2009). Two antibiotics, ampicillin and streptomycin, were also found to stimulate the responses of a non-enzymatic antioxidant, malondialdehyde (MDA), and three antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in *M. aeruginosa*, which were related to their inhibitory effects on both algal growth and microcystin-production (Qian et al., 2012a,b). The above studies suggested that antioxidant responses may be a promising route for investigating the interaction mechanisms between antibiotics and *M. aeruginosa*. To date, studies on the effects of other antibiotics on the antioxidant systems of *M. aeruginosa* are still rare.

Further, antioxidant responses have proven to be related to the degradation of exogenous chemicals in algae, especially for antioxidants involved in the phase II transformation process, such as glutathione (GSH) and glutathione S-transferase (GST) (Field and Thurman, 1996; Liu et al., 2010). Yang et al. (2002) suggested that GSH conjugation was one of the principal mechanisms involved in the degradation of 2,4-dichlorophenol by diatom. Lei et al. (2003) reported that algal species with greater GST activity showed higher pyrene metabolism ability. Wang and Xie (2007) found that increased levels of SOD, GST and GSH were coupled to the degradation of nonylphenols in *M. aeruginosa*. Though the antioxidant responses to certain antibiotics have been observed in *M. aeruginosa* (Qian et al., 2012a,b), the degradation of antibiotics by *M. aeruginosa* has not been reported.

Spiramycin and amoxicillin were selected as target chemicals in the present study, which have been reported to affect the algal growth and the production and release of microcystins in *M. aeruginosa* (Liu et al., 2012). The responses of antioxidant enzymes (including SOD, POD, CAT and GST) and non-enzymatic antioxidants (including MDA and GSH) in *M. aeruginosa* following exposure to target antibiotics, as well as the degradation of target antibiotics by *M. aeruginosa* were investigated, in order to further interpret the interaction effects and mechanisms between target antibiotic contaminants and *M. aeruginosa*.

2. Materials and methods

2.1. Reagents and materials

Spiramycin, amoxicillin and sulfathiazole-d_4 were purchased from Sigma-Aldrich, Inc. (Shanghai, China). All of the stock solutions were prepared in methanol and stored at −20 °C prior to use. An axenic strain of *M. aeruginosa* (PCC7806) was supplied by the Pasteur Culture Collection of Cyanobacteria (Paris, France) and was cultured axenically during the whole experimental period. Oasis HLB glass cartridges (200 mg, 5 ml) were obtained from Waters Corporation (Massachusetts, USA). Visiprep twelve-port SPE Vacuum Manifold was supplied by Supelco (Bellefonte, USA). Motic AE30 inverted microscope was purchased from Motic Optical (Xiamen, China). All the glass wares used for the determination of antibiotics were previously washed with tap water, Milli-Q water, methanol and Milli-Q water again, and finally dried at 550 °C for 4 h before use. All the experimental apparatus used to culture *M. aeruginosa* were sterilized by autoclaving at 121 °C for 20 min before use. All organic solvents, suitable for liquid chromatography-mass spectrometry (LC–MS) analysis, were provided by Merck & Co., Inc. (Shanghai, China).

2.2. Culture of M. aeruginosa

M. aeruginosa was pre-cultivated under static condition for two weeks with sterile BG11 medium (Rippka et al., 1979) at 25 ± 1 °C, under a 16:8 light:dark cycle provided by cool white fluorescent lights at an intensity of 40 μmol photons/m^2/s. After pre-cultivation, algal cells reaching the exponential phase of growth were collected by centrifugation (4000 g, 4 °C, 5 min) and used as the inoculums for the exposure test and the degradation test.

In the antibiotics exposure test, 1000 ml Erlenmeyer flasks, each containing 500 ml of the sterile BG11 medium, were spiked with different concentrations of target antibiotics, each in triplicate. The final concentrations of methanol in the test media were below 0.01% (v/v). Another set of flasks containing sterile BG11 media spiked with 0.01% (v/v) methanol and without antibiotics, also in triplicate, was prepared as the control. Algal cells were inoculated into each flask at an initial cell density of 4×10^5 cells/ml, which were counted under inverted microscope. After inoculation, the flasks were closed with sterile cotton stoppers and cultured in the same condition as the pre-cultivation for 7 days. The flasks were shaken well every 8 h and before each sampling.

During the 7-day exposure, target antibiotics were replenished every 12 h to maintain a stable exposure dose. The amount of target antibiotics and time required for replenishment were based on the results of a preliminary test determining the stability of test antibiotic concentration in each culture media. Test concentrations of antibiotics were found to decline with time in preliminary test, probably due to photodegradation and volatilization. Regular replenishment could make sure that the determined test concentrations deviated within an acceptable range (from −10% to +8%) compared with their nominal values.

The experimental set-up for the degradation test was similar to that for the antibiotics exposure test, except for the following: (i) nine 1000 ml Erlenmeyer flasks, each containing 500 ml of the sterile BG11 medium inoculated with *M. aeruginosa*, were prepared per chemical per concentration, (ii) target antibiotics were only spiked at the beginning of the test with no replenishment during the whole exposure period, and (iii) nine flasks with culture medium but without algae (abiotic control) were also prepared per chemical per concentration for measuring the abiotic loss of target chemical from the culture medium.

2.3. Analysis of antioxidant responses

In the antibiotics exposure test, the culture medium was aseptically sampled from each flask after 1, 4 and 7 days of exposure, respectively. The algal cells from 50 ml of culture medium were harvested by centrifugation at 2000 g at 4 °C for 10 min, re-suspended in 1.5 ml of phosphate buffer (100 mM, pH = 7.4) containing 1% (w/v) of polyvinylpyrrolidone and homogenized on ice by an ultrasonic cell pulverizer at 200 W with total time of 5 min (ultrasonic time: 2 s; rest time: 8 s). The homogenate was then centrifuged at 12,000 g at 4 °C for 10 min. The supernatant was used to determine the contents of total protein and non-enzymatic antioxidants and the activities of antioxidant enzymes. Total protein was determined according to the method of Braford (1976) using bovine serum albumin (BSA) as the protein standard. The SOD activity was determined by the nitro blue tetrazolium (NBT) method (Bayer and Fridovich, 1987). One Unit of SOD activity was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibited NBT reduction. The POD activity was determined according to Kwak et al. (1995). One unit of POD activity was defined as the amount of enzyme required for the formation of purpurogallin from pyrogallol in 20 s at pH 6.0. The CAT activity was determined according to the method of Góth (1991). One unit of CAT activity was defined as the amount of enzyme which degraded 1 μmol H_2O_2 per minute at 37 °C. The GST activity was determined as described by Habig and Jakoby (1981). One unit of GST activity was determined as the amount of enzyme that catalyzed the formation of 1 μmol of S-(2,4-dinitrophenyl)-glutathione per minute at 37 °C. The GSH content was measured by the 5,5′-dithiobis-(2-nitrobenzoic acid)-glutathione reductase (DTNB-GR) recycling assay (Anderson, 1985). The MDA content was quantified based on its chromogenic reaction with thiobarbituric acid as described by Janero (1990). The activities of the antioxidant enzymes and the contents of non-enzymatic antioxidants in *M. aeruginosa* were expressed in units per mg of protein (U/mg protein) and μg/mg protein, respectively, in order to eliminate the measurement deviation caused by varied extraction efficiency of protein.

2.4. Analysis of target antibiotics

In the degradation test, the culture medium was also sampled after 1, 4 and 7 days of incubation, respectively. Three flasks, each containing 500 ml culture medium, were sampled each time to provide triplicate samples for each chemical at each test concentration at each exposure time. The 500 ml culture medium was separated from the algal cells by filtering through a Whatman GF/B filtration membrane (1 μm). For the determination of antibiotic concentration in the culture medium, the filtrate was mixed with 2 ml of 5% (w/v) ethylene diamine tetraacetic acid (EDTA) and Milli-Q water to obtain a final volume of 1000 ml, and then extracted with an Oasis HLB glass cartridge at a flow rate of 2 ml/min. The algal cells harvested by the filtration membrane were freeze-dried, extracted with 10 ml of acetonitrile under sonication and centrifuged at 4000 g at 4 °C for 20 min to remove the cell debris. The extract was also mixed with 2 ml of 5% (w/v) EDTA and Milli-Q water to make a final volume of 100 ml and extracted with an Oasis HLB glass cartridge to measure the adsorption of target antibiotics in algal cells. The Oasis HLB glass cartridge was conditioned with 6 ml of methanol and 6 ml of distilled water before use. After extraction, the cartridge was washed sequentially with 5 ml of Milli-Q water and 5 ml of 5% (v/v) aqueous methanol. The cartridge was finally eluted with 8 ml of methanol after it had been dried under a vacuum for 2 h. The elution solvent was blown down to dryness under a gentle flow of nitrogen, spiked with 50 ng of sulfathiazole-d_4 as an internal standard, dissolved with 100 μl of methanol, and subjected to a liquid chromatography-tandem mass spectrometry (LC–MS/MS) system.