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Effects of arginine on the growth and microcystin-LR production of *Microcystis aeruginosa* in culture

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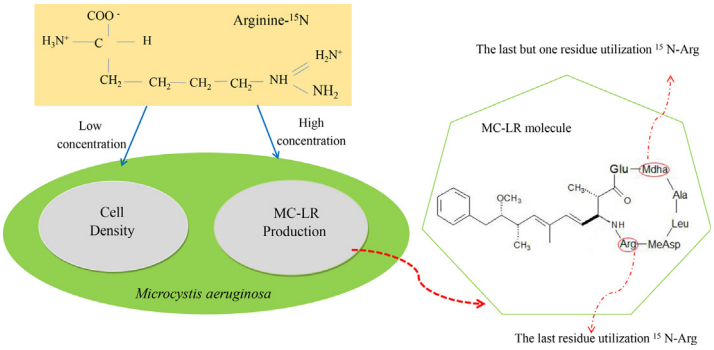
HIGHLIGHTS

- Arg promotes the growth and MC-LR production of *M. aeruginosa*.
- Arg tended to be incorporated easily into amino acid residues of MC-LR.
- Arg in MC-LR was the last amino acid residue to incorporate ^{15}N from ^{15}N-Arg.
- Mdha was the last but one amino acid residue to do so.

GRAPHICAL ABSTRACT



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ABSTRACT

Although toxic cyanobacterial blooms and their toxins threaten drinking water and ecology and are promoted by nutrient loading, the precise nutrient regime that increases cyanobacterial populations and toxin production is poorly understood. Here, the influences of arginine (Arg), as a common amino acid with high nitrogen content, on the growth and microcystins (MCs) production of *Microcystis aeruginosa* (*M. aeruginosa*) were investigated by an isotope method (^{15}N). The results showed that the biomass and production of microcystin-LR (MC-LR) increased with an increase in initial Arg concentrations in the range of 0.3–1.4 mmol-N L^{-1}, whereas a higher Arg concentration (3.6 mmol-N L^{-1}) inhibited the growth. MC-LR on different days (days 0, 6, 12, and 18) was detected by liquid chromatography with tandem mass spectrometry (LC-MS/MS) after incubation with ^{15}N-Arg. The MC-LR molecular weight increased from 995 to 1004 with 100% relative abundance with 10 ^{15}N atoms bound by the Adda, Arg (4 ^{15}N), Glu, Mdha, Ala, Leu, and MeAsp residues on day 18. It seems that there was a sequential order when *M. aeruginosa* assimilated Arg to synthesize MC-LR. The Arg residue in the molecule of MC-LR was the last one to be labeled by ^{15}N from ^{15}N-arginine. This study not only presents a deeper insight into the biosynthesis of free amino acids that are incorporated into MCs but also reminds us of the potential risk caused by Arg, which should arouse concerns.

1. Introduction

Harmful algal blooms (HABs) have been recognized as an emerging issue causing human health problems in the late 20th century. It is becoming increasingly clear that almost every part of the world depending

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on surface drinking water has or will encounter problems with toxic cyanobacteria in its drinking water system (Prakash et al., 2009; Gallo, 2014). *Microcystis aeruginosa* (*M. aeruginosa*) is one of the most common cyanobacteria in fresh water and can produce toxins known as microcystins (MCs). MCs are not only posing a serious threat to the safety of drinking water and ecology (Song et al., 2007; Scholz et al., 2017) but also can bioaccumulate in the food chain (Isaacs et al., 2014). They are hepatotoxins, which may promote liver tumorigenesis by inhibiting a protein phosphatase (Hooser et al., 1991; Mez et al., 1997; Dietrich and Hoeger, 2005; Qiu et al., 2012). During large HABs, the concentrations of MCs can reach the ppm range, suggesting that there is a high probability that humans and animals may be exposed and affected by MCs via drinking water sources (He et al., 2012).

Although over 90 MC variants are known, the most abundant and toxic one is microcystin-LR (MC-LR), which has been identified together with other commonly found MC variants such as MC-LA, MC-RR, and MC-YR found in natural water (Antoniou et al., 2008; Schmidt et al., 2014.) The generalized structure of MCs is described as cyclo (D-Ala-X-D-MeAsp-*Z*-Adda-D-Glu-Mdha-), where MeAsp is D-erythro-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and X and Z are two variable L-amino acids (Miles et al., 2013). Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E-dienoic acid, is a characteristic residue serving as the C_{20} amino acid (Chen et al., 2010). MC-LR, which contains leucine (Leu) at position X and arginine (Arg) at position Z, is one of the most toxic MCs and shows a half-lethal dose (LD_{50}) of 65 μg kg^{-1} i.p. (intra-peritoneal) in mice (Hooser et al., 1989; Falconer, 1992; Towner et al., 2002). In view of MCs' toxicity and the worldwide occurrence of toxic cyanobacterial blooms, deeper research in this field is an urgent need for protecting water resources and environmental safety.

Nutrient loading (derived from anthropogenic activities) in lakes may increase the frequency, severity, and duration of toxic cyanobacterial blooms around the world (Horst et al., 2014; Harke et al., 2015). Different nitrogen forms, both dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON), can be utilized by cyanobacteria (Takamura et al., 1987; Davis et al., 2010; Donald et al., 2011; Steffen et al., 2014; Davis et al., 2015). Nitrate and ammonium are traditionally considered the most important inorganic nitrogen sources (Herndon and Cochlan, 2007). Nonetheless, when HABs occur and DIN decreases to the lowest level, DON becomes the dominant nitrogen present in water (Butler et al., 1979). Under DIN-limited conditions, cyanobacteria may be capable of utilizing DON, especially in the form of dissolved free amino acids (DFAAs) such as alanine (Ala), Arg, and Leu, to synthesize MCs (Yan et al., 2004).

Knowledge about Arg effects on algal growth and toxin production is of great interest because (a) Arg has relatively high nitrogen content (32.2%, $C_6H_{14}N_4O_2$) (Lytle and Perdue, 1981) and (b) it accumulates as a reserve material in cyanobacteria (Simon, 1971). Besides, it is reported that prokaryotic and eukaryotic oxygen-producing photosynthetic organisms store nitrogen as Arg (Llacer et al., 2008). The concentration of Arg is 0.37–0.67 μg L^{-1} in water of Taihu, a large, shallow and eutrophic freshwater lake in China (Yao et al., 2010). It was reported that there is 0.06–0.91 μg L^{-1} Arg in four Swedish lakes Ekoln, Erken, Limmaren, and Vallentunasjon (Bertilsson et al., 2007; Okello et al., 2010). Although the concentration of Arg is not high in water, studies show that Arg utilization may be a significant source of nutrients and energy for cyanobacteria (Bertilsson et al., 2007; Minaeva et al., 2015), and Arg utilization has been studied in various species (Singh and Bisen, 1994; Flynn and Syrett, 1986; Flores and Herrero, 2005; Singh, 2010; Barónsola et al., 2017). Many studies revealed that Arg could be assimilated and can support the growth of cyanobacteria (Rawson, 1985; Flores and Herrero, 2005), but they have not addressed the production of MCs and their relation. It was demonstrated that Arg clearly increases cylindrospermopsin (CYN) production and algae growth (Muenchhoff et al., 2010; Barónsola et al., 2017). Nevertheless, how Arg in the external environment affects cyanobacterial blooms and MC production has not been well studied so far. In addition, MC-LR has an Arg residue as one part of its structure. Therefore, there may be a close relation between Arg and the biosynthesis of MC-LR. On the other hand, the effects of Arg on the growth of cyanobacteria, especially the production of MC-LR, are poorly understood and deserve further research.

HABs can release mixtures of cyanotoxins rather than a single toxin. Therefore, the methods of analysis capable of identifying different analogues concurrently are necessary. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is a powerful method for MC quantification and structural characterization although few studies apply LC-MS/MS analysis to stable-isotope-labeled MCs (Bateman et al., 1995). It is concluded after LC-MS/MS analysis that ^{15}N from ammonia is probably incorporated into the Arg residue. By contrast, ^{15}N from Ala is assimilated into Ala, Leu, iso-linked (2*R*,3*S*)-3-methylaspartic acid, Arg, and certain unusual C_{20} amino acid residues (Yan et al., 2015). Wu further demonstrated in a ^{15}N-labeling experiment that there is selectivity when *M. aeruginosa* incorporates urea nitrogen into its structure. An Ala or Leu residue incorporates one urea ^{15}N atom at first, whereas Mdha is the last amino acid residue to incorporate ^{15}N from ^{15}N-urea (Wu et al., 2015). Nonetheless, there is no research on the incorporation of amino acids, especially Arg, into MC-LR with ^{15}N labeling.

The specific objectives of this study were to systematically describe the growth and MC-LR production of *M. aeruginosa* when cultured in a liquid medium containing Arg and to investigate by LC-MS/MS the roles Arg nitrogen plays in MC-LR production. The main goal addresses the incorporation of free amino acids into MC-LR and gives a deeper insight into the effect of nitrogenous compounds on algae and production of MCs.

2. Materials and methods

2.1. M. aeruginosa and cultures

An axenic strain of *M. aeruginosa* isolated from Lake Dianchi in China was obtained from the Institute of Hydrobiology, the Chinese Academy of Sciences. *M. aeruginosa* can produce only MC-LR (Dai et al., 2008). It was maintained in the BG-11 medium in a climatic chamber (BIC-300, Boxun, Shanghai, China) at 25 °C, with a photon flux of 8–12 μW cm^{-2} and a 12 h photoperiod. The flasks were shaken each day and rearranged randomly to reduce any influences (of minor differences in the light intensity) on the algal growth (Vezie et al., 2002).

2.2. The stable isotopic experiment and mass spectra

Cultures in the exponential phase were concentrated by centrifugation and washed three times with sterile distilled water and then inoculated into the medium without a nitrogen source and grown for a week to exhaust the nitrogen in the cells (Yan et al., 2015). After that, *M. aeruginosa* was added into a series of 2.5 L flat-bottomed bottles containing 2 L of the medium supplemented with ^{15}N-Arg (98 at%, Sigma-Aldrich) at 0, 0.4, 0.7, 1.4, or 3.6 mmol-N L^{-1}. Subsamples were taken at certain intervals after inoculation during the incubation period to determine the cell number, extract the MC, and measure nitrogen in culture supernatants.

On days 0, 6, 12, and 18, subsamples were analyzed by LC-MS/MS to compare the results observed before inoculation, in the initial growth phase, and after long-term cultivation of *M. aeruginosa*. Mass spectra were obtained on a Scientific TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Fisher, USA) with an electrospray ionization (ESI) source. The chromatographic separation was achieved on a C8 column (Aqua, 5 μm i.d., 2.1 × 150 mm, Thermo, USA). The mobile phase was a 35% (v/v) acetonitrile aqueous solution containing 0.05% (v/v) of glacial acetic acid. The flow rate was 300 μL min^{-1}, and the injection amount was 20 μL.