Vol. 82: 105–110, 2018
https://doi.org/10.3354/ame01877

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online September 20

NOTE

Effect of nutrient availability on cylindrospermopsin gene expression and toxin production in *Cylindrospermopsis raciborskii*

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ABSTRACT: *Cylindrospermopsis raciborskii* is a bloom-forming diazotrophic cyanobacterium, the worldwide success of which has been attributed to its phenotypic plasticity and to the presence of ecotypes with different environmental preferences. *C. raciborskii* strains from Oceania and Asia are able to produce cylindrospermopsin, a potent nitrogen-rich cytotoxin. There is no agreement about the biological role of cylindrospermopsin or which environmental factors trigger its production in this species. In the present study, we analyzed the combined effect of nitrate and phosphate availability on the relative expression of a gene involved in cylindrospermopsin synthesis (*cyrA*) and on toxin production in *C. raciborskii* CYP011K. The strain was grown under different combinations of nitrate and phosphate availability. We found that, besides *cyrA* expression being detected in all treatments, the combination of nitrate deprivation and phosphate repletion was the condition significantly inducing *cyrA* expression and toxin production, concomitant with a higher growth rate. Thus, we demonstrated that cylindrospermopsin production in *C. raciborskii* is positively related to nitrogen fixation. We hypothesize that under nitrogen-fixing conditions, the production and export of cylindrospermopsin would be a functional strategy for competition with other phytoplankters via a mechanism involving the inhibition of reduced glutathione synthesis and induction of oxidative damage.

KEY WORDS: *Cylindrospermopsis raciborskii* · *cyrA* · Cylindrospermopsin · Cyanotoxins · Nitrogen availability

INTRODUCTION

Cylindrospermopsis raciborskii is a diazotrophic toxic cyanobacterium (order Nostocales), and its blooms have increased worldwide over the last decade (Bonilla et al. 2012). It is known that strains from Australia, New Zealand and Asia are able to produce cylindrospermopsin (CYN) (Antunes et al. 2015). Its cytotoxic effects include DNA damage, inhibition of protein synthesis, and induction of apoptosis and necrosis (Štraser et al. 2013, Poniedziałek et al. 2015).

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Although diverse environmental factors such as nutrients, temperature and light intensity have been suggested as triggers for the production of cyanobacteria toxins (Neilan et al. 2013), the ecological role of CYN in *C. raciborskii* has yet to be clarified. Since the description of the *cyr* cluster (Mihali et al. 2008), several studies have attempted to elucidate the role of nutrients, light and temperature in CYN production in different species of cyanobacteria. It has been suggested that in the case of the CYN-producer *Aphanizomenon ovalisporum*, nitrogen availability and light intensity determine the transcriptional abundance, usually showing a positive correlation between CYN concentration levels and gene expression. For example, transferring *A. ovalisporum* from standard to nitrogen-depleted medium provokes a decrease in *aoaA* transcript abundance (the homologous *C. raciborskii cyrA*) (Shalev-Malul et al. 2008). It has been suggested by several authors that CYN production is constitutive; however, no gene expression studies addressing this topic have been specifically designed to test this assumption. As Jiang et al. (2012) reported, the CYN-encoding genetic cluster (cyr) of nostocaleans comprises a rearrangement of 2 polycistrons, *cyrA-cyrB-cyrE* and *cyrD-cyrK*, plus a single gene section including *cyrC*. Thus, it is very likely that these 3 operon-like sections would be subjected to different regulation mechanisms and the final product would be modified in response to all of the factors affecting the expression of these sections. For example, Stucken et al. (2014) found that in *C. raciborskii* CS-505, the expression level of genes involved in CYN biosynthesis (*cyrB*, *cyrI*, *cyrJ*) and transport (*cyrK*) was constitutive under different nitrogen sources over time. However, they did not address relative gene expression using housekeeping genes as a reference, so the actual constitutive nature of the transcription cannot be confirmed. Nevertheless, these authors identified putative binding sites for NtcA, a global regulator of nitrogen metabolism within the *cyr* gene cluster. In this sense, it has been recently reported that under nitrogen deficiency, *C. raciborskii* produces larger amounts of total CYN (Yang et al. 2018). These findings, together with the ability of *C. raciborskii* to fix atmospheric nitrogen, point to a potential role of this nutrient in the modulation of gene expression and toxin synthesis.

Regarding the role of phosphate in CYN production, in *A. ovalisporum*, the toxin is a signal for the species to obtain phosphate by inducing alkaline phosphatase in other phytoplankters (Bar-Yosef et al. 2010). In *C. raciborskii*, the role of phosphate in CYN production is less clear: as this species is able to produce cytoplasmic poly-phosphate granules, it shows a high phenotypic plasticity and has a remarkable ability to thrive under low-phosphate conditions (Isvánovics et al. 2000, Amaral et al. 2014). Thus, the species displays a number of strategies for succeeding under nitrate- or phosphate-deprived conditions, which makes predictions about the effect of nutrients on toxin production more challenging. In order to gain insight into the ecophysiology of CYN-producing *C. raciborskii*, we analyzed the effect of nitrate and phosphate availability on CYN production by assessing *cyrA* expression and toxin concentrations. Under the assumption that CYN would be related to nutritional conditions, we hypothesized that nutrient deprivation (either nitrate or phosphate) would trigger the expression of the genes involved in toxin synthesis, resulting in a higher production of CYN in *C. raciborskii*.

MATERIALS AND METHODS

Culture conditions

The CYP011K strain was provided by Humpage, Australian Water Quality Center, Australia, and has been used in many published works. It was originally isolated by Dr Peter Baker from Julius Lake, Mount Isa, Queensland, Australia. The strain was maintained in batch regime with nitrate (N)-free BG11 medium containing phosphate (P) (hereafter –N+P condition) (Stanier et al. 1971). One month before starting the experiments and gene expression analysis, the culture was transferred to 4 nutrient treatments: N- and P-replete (hereafter +N+P), N-deprived and P-replete (–N+P), N-replete and P-deprived (+N–P), and both N- and P-deprived (–N–P) (Table 1). The treatments were run in semi-continuous mode with a 1:1 dilution with modified BG11 medium twice a week (approximately nine 1:1 culture dilutions) as follows. To achieve the +N+P treatment, the original –N+P culture was diluted 1:1 each time with N-full BG11 fresh medium. For both –P treatments (+N and –N), an aliquot of the original culture (–N+P) was diluted in P-free BG11 (–N and +N) fresh medium to a final concentration of 10 µmol l^{-1} total P. For the next instance of medium renewal and thereafter, a half volume was substituted with fresh BG11 medium

Table 1. Concentration of nutrients in the medium, nitrogen (N) and phosphate (P) cell quotas, molar N:P ratios and growth rates in each treatment. DW: cell dry weight

Treatment	Nitrate as $NaNO_3$ (mmol l^{-1})	N cell quota (µg N mg^{-1} DW)	Phosphate as K_2HPO_4 (mmol l^{-1})	P cell quota (µg P mg^{-1} DW)	Molar ratio N:P	Growth rate (µ, d^{-1})
+N+P (control, nutrient-replete)	17.60	67 ± 9	0.23	15.0 ± 2.0	9.9	0.61 ± 0.13
+N–P (P-deprived)	17.60	56 ± 17	0.01	4.0 ± 1.0	31.0	0.25 ± 0.14
–N+P (N-deprived)	No addition	46 ± 12	0.23	7.5 ± 1.0	13.6	0.58 ± 0.36
–N–P (N&P-deprived)	No addition	30 ± 1	0.01	3.5 ± 0.5	19.6	0.59 ± 0.29

containing 10 µmol l^{-1} K_2HPO_4 (Amaral et al. 2014). P deficiency was confirmed by the stationary growth phase observed between dilutions and a P concentration below analytical detection limits (Table 1). Cultures were grown at 26°C and 80 µmol photons m^{-2} s^{-1} with daylight fluorescent tubes (16 h:8 h light:dark photoperiod). Cultures were maintained in 1 l borosilicate bottles continuously bubbled with pre-filtered (0.45 µm) saturated humid air, pre-bubbled in ultrapure sterilized water and monitored daily by optical density (OD) measurements at 750 nm (Thermo Scientific, Evolution 60 LC). All culture conditions were run in triplicate. Samples for RNA extraction, toxin concentration determination and nutrient quotas were taken from each nutrient growth condition at the fifth day of 1:1 culture dilution. Nutrient cell quotas were determined by filtering triplicate aliquots of cell culture collected in GF/F filters and measured as total nitrogen and total phosphorus (filters pre-soaked with Milli-Q water and soaked after filtration with N- and P-free BG11), and filter blank subtracted, according to Valderrama (1981). The same filtration procedure was applied for dry weight determination. Growth rates (d^{-1}) for each replicate were calculated as the slope of an adjusted linear regression of OD (in ln) versus time (in days) during the exponential phase of the cultures.

cyrA expression levels

Filaments from each flask were harvested by filtration on 0.22 µm pore size sterile filters (filtration lasted less than 10 min) and the relative expression of *cyrA* was assessed (Muenchhoff et al. 2010). Briefly, cyanobacterial biomass was chemically lysed (Piccini et al. 2011) and centrifuged at 8000 × *g* for 10 min, and total RNA was purified using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen). Retrotranscription of the RNA was performed by QuantiTect Reverse Transcription (Qiagen) and the obtained cDNA was diluted (1/10) to use as target in quantitative real-time PCR (qPCR). The qPCR reactions were performed using the QuantiTect SYBR Green PCR kit (Qiagen), 0.3 µM of each primer and 2 µl of the diluted cDNA in a final volume of 20 µl. The primer pairs were cyrAf (5′-GAA CCG CCA AAC TCA AAG AC- 3′) and cyrAr (5′-CCG CTT CAT GAG TTG CTA GA-3′) for the *cyrA* gene (amplicon size of 80 bp), and the gene for the cyanobacterial small ribosomal subunit (16S rDNA) was used as a reference gene using primers 740f (Hugenholtz et al. 1998) and 809r (Jungblut et al. 2005). Cycling conditions were those recommended by the kit manufacturer using an annealing temperature of 52°C in a Rotor Gene 6000 (Corbett Research). qPCR samples from each treatment flask were run in triplicate. The relative abundance of transcripts was calculated using the nutrient-replete condition (+N+P) as control (Pfaffl 2001).

Determination of CYN concentration by ELISA

At the end of the incubation, total CYN concentration (intracellular and extracellular) was determined by ELISA (Abraxis).

Data analyses

To compare *cyrA* relative expression and CYN concentration between treatments, data were first transformed (log+1) to fulfill normality requirements (Kolmogorov-Smirnov normality test) and then a one-way ANOVA was performed. Multiple comparisons were performed using Dunnett's or Tukey's pairwise multiple comparison post hoc tests, when necessary.

RESULTS AND DISCUSSION

We found a significant increase in *cyrA* expression in the –N+P treatment relative to the control (+N+P) (ANOVA, $p \leq 0.05$), whereas in the treatments with P deficiency, the relative expression of *cyrA* decreased below that of the control treatment (relative expression < 1) (Fig. 1A). According to cell quotas, in both –P treatments, P-depleted conditions were achieved (Table 1). The growth rate in the –N+P treatment was similar to that in the control treatment (+N+P) ($p > 0.05$), which was reflected in slight, non-significant differences in N cell quota between both treatments (Table 1). Therefore, we conclude that in the –N+P treatment, cells probably obtained N from biological fixation. We also found that the total CYN concentration was significantly higher in the –N+P treatment when compared to the control (ANOVA, $p < 0.001$; Fig. 1B), in agreement with the change in gene expression, indicating that *cyrA* transcription and toxin synthesis were coupled. Similar findings, but only by assessing toxin concentration, were reported by Saker & Neilan (2001), who found the highest concentrations of CYN in cultures grown without a fixed N source. Moreover, Yang et al. (2018) also found higher CYN concentrations at lower N levels, and stated that under this condition, CYN would be kept intracellular. However, these studies did not address the induction of gene expression, and defined intracellular toxin as that measured from filter-retained biomass. Taken together, these findings suggest that in *C. raciborskii*, the CYN production would be triggered by environmental conditions inducing nitrogen fixation, such as both –N conditions in the present study.

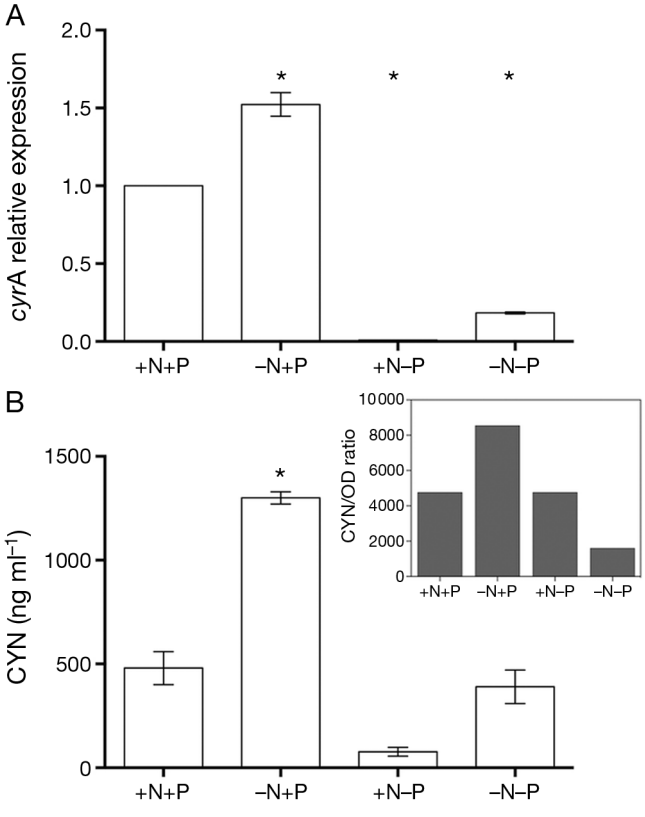

Fig. 1. (A) Relative expression of *cyrA* at the end of nutrient treatments using Pffafl's (2001) formula. Gene expression quantified in cultures under nutrient-replete conditions (+N+P) was used as control. (B) Total concentration of cylindrospermopsin (CYN) at the end of the incubation treatments. Inset shows the CYN concentration normalized by the optical density (OD) reached in all of the assayed conditions. Mean ± SD are shown. Asterisks indicate significant differences related to the control treatment (ANOVA, Tukey post hoc test, $p \leq 0.001$). See Table 1 for treatment details

A competition-related role for CYN has been described by Bar-Yosef et al. (2010) for *A. ovalisporum* related to P availability. In the case of *C. raciborksii*, its known phenotypic plasticity in relation to P availability would rule out a P-scavenging role for CYN. Yang et al. (2016) reported that CYN production and *cyr* gene expression in *C. raciborskii* were not directly related to P concentration but to the growth rate. Similarly, our results showed that under P deprivation and in the absence of nitrate, *C. raciborskii* CYP011K growth rates and *cyrA* relative expression were the lowest observed. This implies that long-term P starvation would negatively affect *C. raciborskii* growth more than inorganic N starvation, probably owing to the ability of this species to fix atmospheric N. In this context, slow-growing, P-starved cells would invest less energy in CYN synthesis. Likewise, Burford et al. (2014) found that cell division rates and CYN cell quotas of phytoplankton populations dominated by *C. raciborskii* were significantly higher when treatments were maintained under P-replete conditions. Conversely, Willis et al. (2015) found a linear relationship between the CYN cell quota and the growth rate for 3 *C. raciborskii* strains, but growth rate decreased with increasing P addition. Hence, the current evidence about the role of P in the growth of and CYN production by *C. raciborskii* is quite contradictory. In the present study, the significantly higher *cyrA* expression and CYN concentrations found in the –N+P treatment was remarkable and seemed to be related to an N-fixing situation combined with a high growth rate. These are conditions that can be found in natural water bodies when blooms develop, although the relevant mechanism has yet to be elucidated.

Several NtcA binding sites have been predicted for the *cyr* cluster, for the *hypF*, *cyrA*, *cyrB*, *cyrE*, *cyrJ* and *cyrK* genes (Stucken et al. 2014). This transcriptional regulator is required to express different ammonia-repressible genes, and in *Anabaena* sp. activates the gene expression for heterocyst development (Frías et al. 1994). It is known that NtcA responds to the N regime through signaling by the internal levels of 2-oxoglutarate, a C:N cell balance indicator. Thus, the evidence suggests that when *C. raciborskii* grows under deprivation of dissolved inorganic N, the shift in the C:N balance provokes an accumulation of 2-oxoglutarate, which induces the binding of NtcA to those genes harboring NtcA-binding boxes, such as those from the *cyr* cluster, triggering their transcription (including the putative transporter *cyrK*) to start CYN synthesis and export. Moreover, it has been shown that CYN inhibits the synthesis of reduced glutathione (Runnegar et al. 1995, Humpage et al. 2005), which is a key molecule involved in cell resistance to oxidative stress (such as protection from rective oxygen species and peroxides). In this context, CYN production and rapid export could induce oxidative damage in other phytoplankters. This implies that CYN could be a competition-related metabolite; however, more focused studies need to be performed in order to confirm this hypothesis.

Acknowledgements. This work was supported by PEDECIBA-Biología, ANII_PR_FCE_2009_1_2330 and FCE2007_353 (ANII, Agencia Nacional de Investigación e Innovación). We thank Beatriz Brena for performing the ELISA analysis.

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Editorial responsibility: Rutger de Wit, Montpellier, France

Submitted: July 26, 2017; Accepted: May 16, 2018
Proofs received from author(s): August 20, 2018