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Microcystin Production and Regulation under Nutrient Stress Conditions in Toxic *Microcystis* Strains

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Microcystin is a common and well-known cyanobacterial toxin whose intracellular role is still under investigation. Increasing knowledge on microcystin gene expression and regulation can contribute to the understanding of its putative cellular function. In this work, reverse transcription-quantitative PCR (RT-qPCR) was used to investigate the transcriptional response of the *mcyD* gene to nitrogen (nitrate and ammonium) and phosphorus limitation in two toxic *Microcystis* strains. The existence of a direct correlation between transcripts of *mcyD* and *ntcA* genes was also identified. In previous studies, NtcA (global nitrogen regulator) has been described as a potential component in the control of microcystin biosynthesis. This research showed that stress agents linked to nutrient deprivation could lead to a significant increase of microcystin production in both strains studied. The more toxic strain proved to be more resistant to nutrient limitation. The similar outcomes of *mcyD* regulation observed for all nutrients suggest that this response can be linked to oxidative stress of cells undergoing adverse growth conditions.

Bloom-forming cyanobacteria occur worldwide and produce toxins that may be harmful to humans and animals (1). Earlier studies suggested that microcystin net production depends primarily on cellular growth rate, while environmental conditions would affect microcystin production rather indirectly, via the cellular growth rate itself (2, 3). Other research papers, however, tried to verify the influence of environmental factors on microcystin production (4, 5, 6). Controversial results were generated using direct measurements of intracellular toxins, mostly because of differences in culturing techniques, growth conditions, as well as experimental design and analyses (7). Recently, some authors (8) showed a significant effect of environmental factors on microcystin production, and they observed that the effect was independent of influences on growth rate. For phosphorus and irradiance, intrinsic growth rate and microcystin production coefficient were inversely correlated.

Investigations about the role and function of microcystin are still widely discussed. Several potential roles were suggested, as, for example, iron chelator (siderophores) (2, 9), defense mechanism (10), photosynthesis or other light-related processes (11), and intercellular intraspecies communication (12). With the discovery that microcystin was not produced by ribosomes and the existence of a specific gene set, called *mcy* (13), there was a significant increase in studies on factors that may regulate microcystin production, contributing to the understanding of ecological questions about microcystin function (14, 15, 16, 17).

Recent studies indicated an intracellular function related to protein ligand (18) and found that several proteins of toxic and nontoxic strains were differentially expressed as a result of strain toxicity (19). It was also shown that the addition of hydrogen peroxide had less detrimental effects on toxic *Microcystis* strains than on nontoxic ones (20), suggesting protection by microcystin. Another report (21) demonstrated the increase of microcystin synthesis, but not of the gene transcription for toxins, in cells exposed to severe limitation of iron. Also, experiments run under iron deficiency (22) found increasing transcription levels of the *mcy* gene and of microcystin synthesis. When testing nitrogen limitation, Sevilla et al. (23) did not see any effect of nitrate reduction in the production of microcystin. However, Ginn and Neilan (24) showed that the microcystin synthetase gene transcription was responsive to nitrogen, increasing under nitrogen limitation. These authors also discovered that the *mcyABC* and *mcyDEFGHIJ* (hearafter called *mcyA*/*D*) promoter region has binding sites for the universal regulator of nitrogen (NtcA), indicating its importance for the regulation of microcystin production.

As primary producers, cyanobacteria need nitrogen and phosphorus as essential macronutrients, and changes in their concentration observed in the environment have several physiological effects. Phosphorus is important for the cellular synthesis of nucleic acids and membrane phospholipids, as well as for energy transfer through tri- and biphosphorylated nucleotides (25). In aquatic environments, dissolved inorganic phosphorus is biologically available as orthophosphate (26) and can easily become limiting. Nitrogen assimilation is ruled by the available nitrogen source (27). Inorganic forms like ammonium and nitrate are the most common in water and can both be assimilated by cyanobacteria following different pathways (28). As all planktonic organisms, cyanobacteria live in permanently changing environments, undergoing seasonal challenges, and may have to overcome periods of nutrient limitation. Since it triggers physiological changes, nutrient limitation can also affect the mechanisms of toxin production. A better knowledge of these mechanisms may help to evaluate the importance of cyanotoxins for cyanobacteria. In addition, it can provide tools that will improve the environmental control of these organisms.

Many studies used *Microcystis aeruginosa* PCC 7806 as a model organism. However, different authors already advised that the strains present in nature represent numerous ecotypes able to adapt and to survive in a specific environment (29, 30). Among

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TABLE 1 Sequences of the primers used in this study for *mcyD*, *cpcB*, *ntcA*, and 16S rRNA

Primer	Type[a]	Sequence (5′ to 3′)	Reference
q-mcyD	F	GCATCTTCTAAAGAAAAGACTCC	47
q-mcyD	R	TATTCCCCAAGATTGCCATAATTT	47
QcpcB	F	GGTATCACCCCCGGCGATTG	This study
QcpcB	R	GCAGCAGCAGCGCGATCGAAGTA	This study
QntcA	F	TGCAGGGTTTGTCCTCGCGG	This study
QntcA	R	CCCGGATGCCATCGGTGGTG	This study
16S-rRNA	F	TGCGTAGAGATTGGGAAGAACATC	23
16S-rRNA	R	GCTTTCGTCCCTGAGTGTCA	23

[a] F, forward; R, reverse.

others, one work (21) drew attention to the dangers of using just this strain as a model for the entire *Microcystis* group, since strains may present specific acclimation processes under stress conditions. Therefore, to study the mechanisms of microcystin production and their effects on metabolism, it would be meaningful to investigate new strains and to follow their response to particular surroundings.

The aim of this study was to measure changes in *mcyD* gene transcription in response to nitrogen and phosphorus limitation in two *Microcystis* strains, isolated from Brazilian water bodies. Both strains are toxic, but they produce different amounts of microcystin. We also wanted to prove the existence of a correlation between *mcyD* and *ntcA* transcripts, previously found for *Microcystis* PCC 7806. The 16S rRNA gene was used as the reference (endogenous control) and *cpcB* as the control (known response). The results of this research can contribute to the existing literature that will help with the understanding of the ecological importance of microcystins for cyanobacterial cell metabolism.

MATERIALS AND METHODS

Growth conditions and experimental design. Two *Microcystis aeruginosa* toxic strains, strains Ma19 and Ma26, were provided by the Phycology Laboratory of the Universidade Federal de Minas Gerais (Belo Horizonte, Brazil). Both strains had been isolated from Brazilian reservoirs. For these experiments, cells were grown in a WC medium (31) under batch conditions, at a temperature of 24°C, 40 μmol of photons m^{-2} s^{-1}, and a 12-h light and 12-h dark photoperiod. When growing on complete WC medium, strain 26 is approximately 10 times more toxic than strain 19 (previous observations; see Results). For the nitrogen-limited experiments, strains were grown under high nitrogen supply (1 mmol $liter^{-1}$ of N-NO_3^- or 0.2 mmol $liter^{-1}$ of N-NH_4^+, original N concentration in WC medium) and two nitrogen-limited conditions: 0.1 and 0.01 mmol $liter^{-1}$ of N-NO_3^- or 0.02 and 0.002 mmol $liter^{-1}$ of N-NH_4^+ (respectively, 1/10 and 1/100 dilution). A similar procedure was performed for the phosphorous experiments. The original concentration was 0.050 mmol $liter^{-1}$ of P-PO_4^-, and limitation was 0.005 mmol $liter^{-1}$ and 0.0005 mmol $liter^{-1}$ (1/10 and 1/100 of the original concentration).

Strains were inoculated in 200 ml of medium at the three different nutrient levels and grown under these conditions for about 1 week, for acclimatization. After this period, subsamples containing approximately 20,000 cells ml^{-1} were reinoculated in their respective medium, and the experiment started. Experiments were finalized after 6 days, during the exponential growth phase. Aliquots of 20 ml of the culture samples were harvested on glass fiber filters (GF/F), and filters were kept frozen (−80°C) until analysis. During the experiments, small aliquots (2 ml) of cultures were removed every 2 days to estimate the growth rate. Cell counting was carried out in a Fuchs-Rosenthal hemocytometer. All the experiments were done in triplicate (three independent subcultures for each treatment, growing under the same experimental conditions) and repeated at least two times. The entire experimental procedure was performed under completely axenic conditions.

Chlorophyll *a* and microcystin measurements. Chlorophyll *a* was extracted using hot ethanol, measured according to Nusch (32), and quantified on a spectrophotometer at a 665-nm wavelength.

Microcystin was extracted with 75% (vol/vol) methanol. The analyses were performed with an Abraxis-ADDA enzyme-linked immunosorbent assay (ELISA) kit, by following the manufacturer's instructions. Plates were analyzed on a microtiter plate ELISA reader (Bio-Tek, Elx 800) at 450 nm within 15 min after the addition of the stop solution.

Isolation of mRNA and RT-qPCR. RNA was extracted after cell lysis with TES (25% [wt/vol] sucrose, 100 mM EDTA, and 50 mM Tris-HCl, pH 8.0). Samples were left for 2 h at 4°C and then, after addition of lysozyme (5 mg/ml), for 1 h at 37°C and, after addition of proteinase K (100 μg/ml), for 1 h at 60°C; finally, TRIzol (Invitrogen) was added according to the manufacturer's recommendations. Total RNA was resuspended in 50 μl of DEPC-H_2O, and RNA was treated with 1 U/μg of

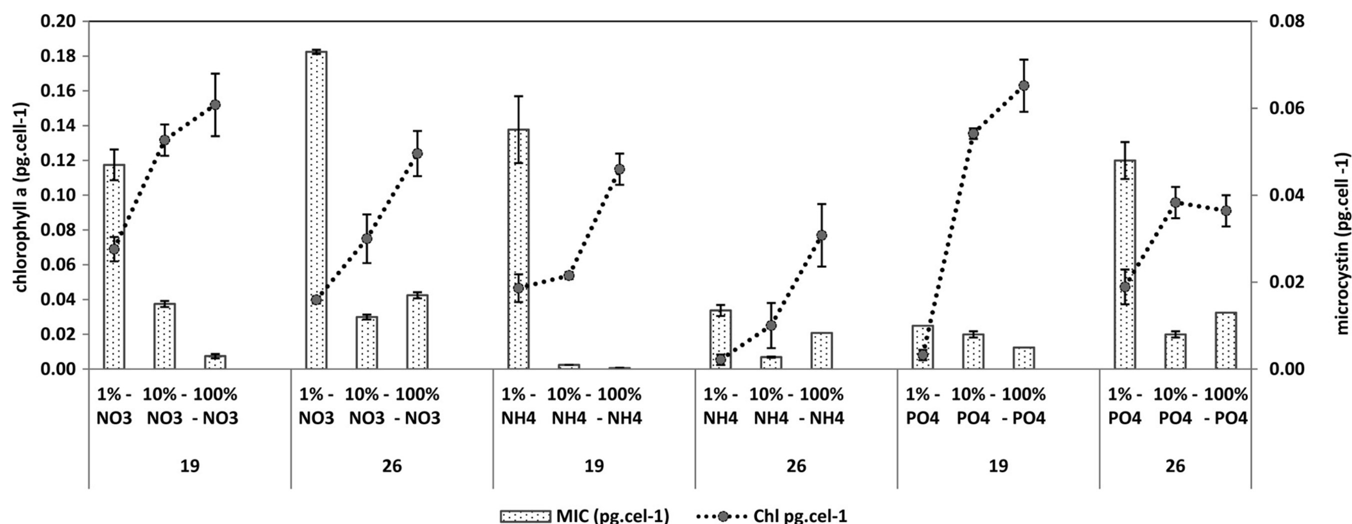

FIG 1 Chlorophyll *a* and microcystin content per cell, for each strain (19 and 26) at three nutrient concentrations, in the nitrate, ammonium, and phosphate experiments. Bars represent standard errors of the means.

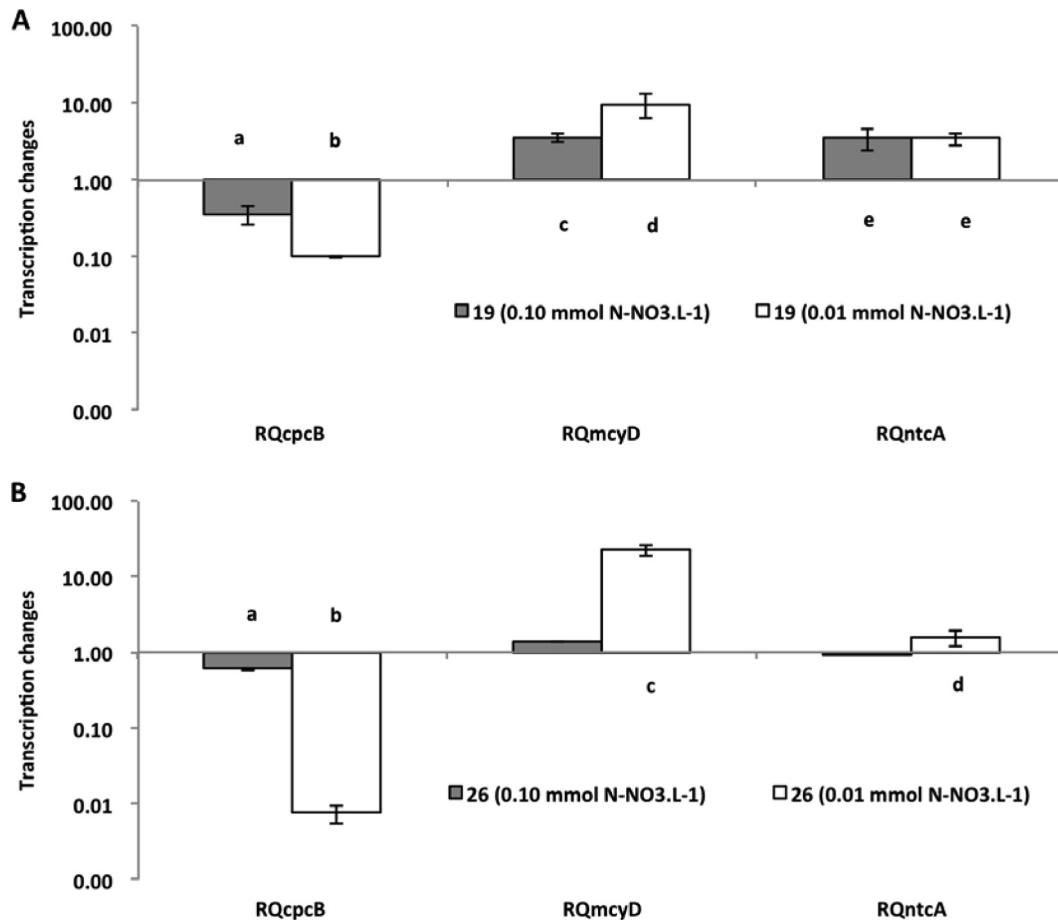

FIG 2 Relative quantification of *cpcB*, *mcyD*, and *ntcA* gene expression in the nitrate assay for two cyanobacterial strains (19 and 26). The 1 mmol liter^{-1} concentration was used as a standard (control) to estimate changes in gene expression under limited conditions. Bars indicate standard errors. Different letters above the bars indicate significant differences ($P \leq 0.05$), when values were compared to the control and among themselves.

DNase (Promega) at 37°C for 30 min. The reaction was stopped by the addition of a stop solution and by heating for 10 min at 65°C. Removal of DNA traces was confirmed by PCR. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was verified on 1% DNase-free agarose gel.

About 500 ng of RNA was used for RT-PCR. The cDNA was generated using a High Capacity kit (Applied Biosystems) with RT random primers. Concentrations and PCR cycling conditions were established according to the manufacturer's recommendations.

Real-time PCR was performed using a StepOne system (Applied Biosystems) with 1 μl of cDNA sample, 0.3 μl of each primer (10 pmol μl^{-1}), 5 μl of Power SYBR green I (Applied Biosystems), and sterile Milli-Q water for a final volume of 10 μl. The reactions were done in duplicate or triplicate, and specifications for the PCR cycle followed the manufacturer's guidelines. Primers used in this study are described in Table 1. The new primers were designed with the help of Primer-BLAST tools (NCBI) to amplify 141-bp products for the global nitrogen regulator gene (*ntcA*), 73-bp products for the phycocyanin gene (*cpcB*), and 103-bp products for the microcystin synthetase D gene (*mcyD*). The primer described by Sevilla et al. (22) for the 16S rRNA was used as a housekeeping gene. The primer amplification efficiencies for *ntcA*, *cpcB*, *mcyD*, and 16S rRNA genes were calculated, and they yielded E values of 1.93, 2.11, 1.95, and 1.97, respectively, where an E value of 2 indicates 100% PCR efficiency. Relative quantification of the *ntcA*, *cpcB*, and *mcyD* target genes was compared with the 16S rRNA reference gene and was represented as the change in transcription compared to the result observed under the control conditions (high nutrient). According to Pfaffl (33), threshold cycle ($\Delta C_T = C_T$ target gene – C_T housekeeping gene) values were used for correlation analyses between *mcyD* and *ntcA* genes. The 16S rRNA reference gene was selected because it is known to be a good housekeeping gene in *M. aeruginosa* PCC7806 (14), and it was already used in several other studies.

The analysis of the fluorescent melting curve was performed to determine the amplification melting temperature of the single PCR products in the samples, by gradually increasing the temperature from 70°C to 95°C at a rate of 0.1°C s^{-1}. Fluorescence intensity data were collected continuously and were converted to melting peaks using the LightCycler software (StepOne Software, version 2.0).

Statistical analyses. Statistical analyses were performed by using the general linear model (GLM) using R software (34). Analysis of variance (ANOVA) was used to test for difference among treatments under different nutrient concentrations. Significant difference was accepted when P values were ≤0.05. ANOVA was run on a Gaussian (normal) distribution for most data sets, but in a few cases, according to the data dispersion, the gamma (chi-square) distribution was preferred and shown to be more robust. Contrast analysis was used to verify differences among treatments. Correlation analyses were run between ΔC_T values of *mcyD* and *ntcA* genes for each nutrient treatment.

RESULTS

The treatment with the highest nutrient concentration was always used as a reference for the quantification of the gene expression of

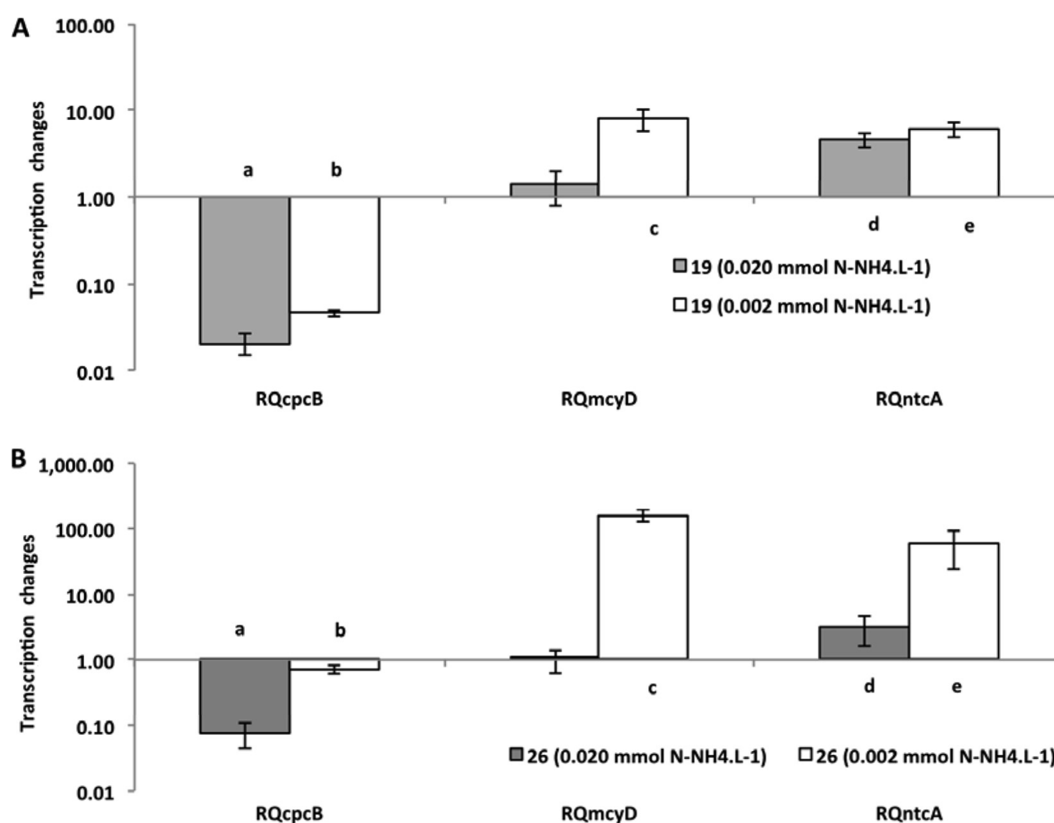

FIG 3 Relative quantification of *cpcB*, *mcyD*, and *ntcA* gene expression in the ammonium assay for two cyanobacterial strains (19 and 26). The 0.2 mmol liter^{-1} concentration was used as a standard (control). For figure explanation, see the legend to Fig. 2.

the treatments with less nutrients. Despite the differences in toxicity of the two strains investigated (strain 26 is approximately 10 times more toxic than strain 19 [previous observations and also Fig. 1]), they performed very similarly, and only small differences were observed between them relative to gene transcription. The *mcyD* gene expression showed changes under all extreme limiting conditions. In the nitrate limitation experiment (Fig. 2), strain 19 had a significant increase in expression level ($P < 0.001$) under both limited conditions, but strain 26 had a significant increase in expression level only under the most extreme condition ($P < 0.001$), when nutrient was reduced 100 times from the original concentration. Interestingly, strain 26 was the strain with 10 times higher content of microcystin and did not show significant changes under the less severe limitation condition. In the ammonium limitation experiment (Fig. 3), an increase of *mcyD* gene expression was observed only at the lowest nutrient level, for both strains (strain 19, $P = 0.020$; strain 26, $P < 0.001$). For phosphate limitation (Fig. 4), both strains had similar responses, with significantly higher expression in the extreme limited concentration ($P < 0.010$).

The response of the *ntcA* gene showed a significant increase in its expression ($P < 0.001$) in all experiments under limitation (Fig. 2, 3, and 4), but individual differences were observed. For nitrate, strain 26 responded only in extreme limitation. Again, it was the more toxic strain 26 that was not affected under less severe limitation.

The analysis of the expression of the *cpcB* gene revealed a significant decrease in the level of transcripts in all experiments ($P < 0.001$) (Fig. 2, 3, and 4). However, once again, the decrease observed in strain 26 in the phosphorus experiment was significant only at the lowest concentration of P-PO_4.

Figure 5 represents correlation analyses between ΔC_T *mcyD* and ΔC_T *ntcA*. All measured values for each nutrient were pooled (all replicates and experimental repetitions), in order to increase the significance of the correlation. The existence of correlation between these two variables was confirmed for all experiments that had been performed under nitrate (Fig. 5A), ammonium (Fig. 5B), and phosphorus limitation (Fig. 5C). All three correlations displayed a significant R^2 value.

As expected, chlorophyll *a* concentration in each culture decreased as the amount of nitrogen or phosphorus supplied to the medium was reduced ($P < 0.001$) (Fig. 1). However, we observed a clear and opposite trend for microcystin content per cell (Fig. 1) for all treatments. For example, at a lower nitrate concentration, a significantly higher concentration of microcystin was measured ($P < 0.001$), showing a direct relationship between nutrient limitation and increase in microcystin.

DISCUSSION

Typically, nitrogen starvation induces physiological responses in the cell, like chlorosis, increased uptake of alternative nitrogen sources, and, once these reach complete depletion, onset of a dormant state (35). In our experiments, we detected the decrease of *cpcB* gene expression (phycocyanin-related gene), of chlorophyll *a* concentration, and of cell growth, and we observed a loss of the culture pigmentation. Chlorosis can induce cells to acquire nitro-

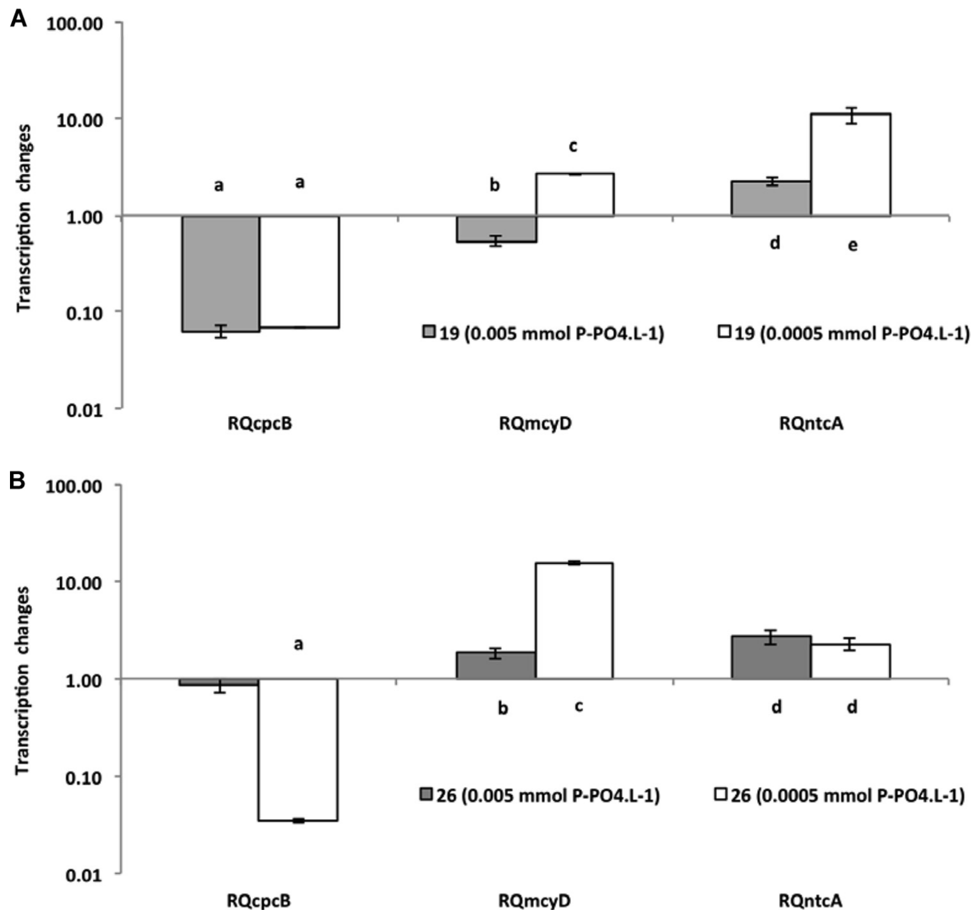

FIG 4 Relative quantification of *cpcB*, *mcyD*, and *ntcA* expression genes in the phosphate assay for two cyanobacterial strains (19 and 26). The 0.05 mmol liter^{-1} concentration was used as a standard (control). For figure explanation, see the legend to Fig. 2.

gen via degradation of the phycobiliproteins, which normally represent a large part of the protein pool in the cells (36) and may comprise up to 60% of total cellular proteins in cyanobacteria (37). In addition, during nutrient starvation, reactive oxygen species (ROS) can be formed, due to the existence of several cross-regulatory reactions between photosynthesis, redox control, and nutrient acquisition, establishing a tight control over the C/N balance in the cell (37). Furthermore, according to Dagnino et al. (38), the loss of pigmentation by discoloration, as we observed for limiting nutrient conditions, is an important sign of the occurrence of oxidative stress. Chlorophyll *a* bleaching was also used by Zilliges et al. (18) as an evidence of oxidative stress, in their case caused by high light conditions and later proved by the addition of hydrogen peroxide. Thus, although no direct measurement of oxidative stress was performed in this study, as previously stated by numerous authors, phenotypic changes, like the observed loss of pigmentation under nutrient limitation, provide strong evidence that the cultures were experiencing oxidative stress.

Stress conditions caused by nutrient deprivation clearly affected *mcyD* transcription and microcystin production in our experiments. Similar trends were found in a recent study, where higher values of microcystin and nodularin cellular content were measured in cells growing under limiting conditions of phosphate and low light (39). Ginn and Neilan (24) also observed an increase in the transcription of the *mcy* gene under conditions of nitrate starvation. However, Sevilla et al. (23) showed that while a decrease of nitrate caused lower cell growth, it did not directly affect *mcy* transcription or toxin production. Nevertheless, the lowest nitrate concentration used by these authors (0.2 mmol liter^{-1}) was not as low as in our experiments or in experiments by Ginn and Neilan, and this is probably the reason for the difference in their results. For example, in this study, we used a nitrate concentration about 20 times lower (0.01 mmol liter^{-1}) than that used by Sevilla et al. (23), and Ginn and Neilan (24), who found results similar to ours, used an entirely N-depleted medium (no nitrate addition). Conflicting results occur mainly because investigations on regulatory mechanisms of toxin production have not yet been standardized and experimental designs and growth controls are frequently different; therefore, outputs tend to be controversial (7).

In recent years, there was a progress in the understanding of the biological role of microcystin, and several studies pointed to potential intracellular functions of this compound. For example, studies done with *mcy* mutants showed differences in pigmentation (40), and toxic and nontoxic strains altered their response to low inorganic carbon concentrations because of the limited ability of the mutant to adapt to low-C conditions (41). The differences we observed between our two strains give further support to the idea of a potential microcystin role under nutrient stress. In fact, the strain with higher toxicity (Ma26) responded with significant

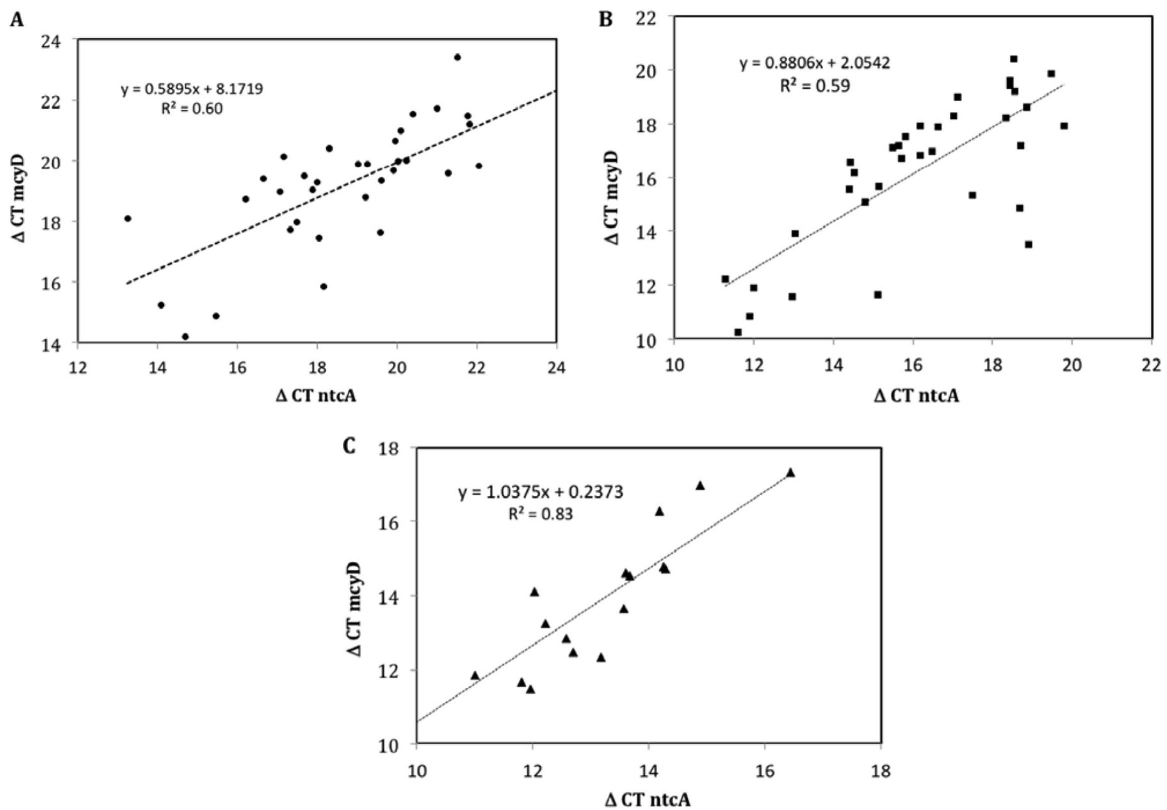

FIG 5 Correlation analyses between ΔC_T values of the *mcyD* and *ntcA* gene expression for all experimental data obtained from the nitrate (A; dots), ammonium (B; squares), and phosphorous (C; triangles) experiments.

mcyD increase only at the lowest nutrient concentration, while the less toxic strain (Ma19) showed an increase of transcripts at both limiting conditions. This may indicate that strain 26 is more resilient to environmental nutrient variations. Investigating the potential relationship of microcystin to increasing fitness, Zilliges et al. (18) also found that a microcystin-deficient mutant was more sensitive to oxidative stress conditions than the wild type. In another study, run under iron limitation and starvation, microcystin-producing cells showed a less pronounced change in iron stress-induced protein A transcript (*isiA*) than nonproducing ones, a finding that was also correlated with the reduced bleaching of the toxic cells under iron-starving conditions (19). Comparing toxic and nontoxic *Planktothrix* strains, Briand et al. (42) observed that microcystin-producing strains had clear advantages against the nontoxic strains under limiting environmental conditions. The lower toxicity of strain 19 seems to increase sensitivity to changes in nutrient supply that was detected by higher cell beaching (data not shown), as well as earlier responses of *mcyD* transcription and microcystin production. Therefore, different toxin content among strains may be relevant to indicate paths of fitness in toxin-producing organisms and may help explain changes in the toxicity of a natural population under shifting environmental conditions, when strains would be selected according to their ability to overcome temporary limitation.

We found that nutrient stress affected *mcyD* transcription, and it was directly correlated with *nctA* gene expression, presenting significant results in all experiments. This correlation can be a strong indication of an intracellular function of microcystin. NtcA activates nitrogen-responsive genes and therefore has been used as a good marker in studies on nitrogen deprivation (43). The autoregulated *ntcA* gene is transcribed at a basal level in the presence of ammonium and increases under conditions of nitrogen stress (24). Ginn et al. (24) have identified regions with similarity to the consensus motifs bound by NtcA in the internal *mcyA*/*D* promoter region in *M. aeruginosa* PCC 7806 and suggested that the microcystin synthetase gene would be responsive to nitrogen. Similar investigation confirmed the affinity of NtcA to promoter regions of *mcyA*/*D* as well as to the regions *mcyE* and *mcyH* (44). These authors observed that the presence of oxoglutarate (2-OG) increased this affinity 2.5-fold. In cyanobacteria, 2-OG is key indicator of the C/N cell balance (27). These results suggest that NtcA activity and microcystin synthesis respond to the equilibrium between C and N metabolism (44). Some authors (45) described the *pkn22* operon, which consists of genes related to iron deficiency and oxidative stress, as also being under the control of NtcA. They suggested that nitrate removal would lead to an electron transfer from ferredoxin to oxygen, generating ROS. Since *ntcA* expression is affected by the cell redox state and NtcA can bind to the *mcy* promoter region, it seems that microcystin may have an important role in the carbon and nitrogen metabolism and in the redox control and perception of redox changes (7).

Changes in the cell redox state are processes that can be intimately linked to the generation of reactive oxygen species and oxidative stress in cyanobacteria (21). Therefore, we suggest that the increase of microcystin observed in our experiments is linked to oxidative stress caused by severe nutrient limitation. By adding

H_2O_2 to a culture in logarithmic growth phase, Schatz et al. (12) induced *mcyB* accumulation. Other reports showed that H_2O_2 stimulated the transcription of the *mcyA* gene and increased the activity of microcystin peptide synthetase (46). Microcystin was shown to bind to proteins under oxidative stress conditions as a possible mechanism to increase fitness of toxic *Microcystis* strains (19). By using high-light conditions and inducing oxidative stress, the researchers observed a specific and covalent interaction of microcystin with several proteins, resulting in their accumulation, suggesting another possible function of this metabolite.

In summary, the results reported here of increasing *mcy* transcription and microcystin production under nitrogen and phosphorus stress conditions are in agreement with some recent reports that point to an intracellular function of microcystin related to oxidative stress. Therefore, in our experiments, higher levels of transcript and metabolite were possibly produced for potential protection of cells under stress. Further studies may help to confirm this putative role of microcystin in the cellular metabolism and to partially elucidate the toxin dynamic observed in the field.

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