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THE INHIBITORY EFFECT OF CALCIUM ON *Cylindrospermopsis raciborskii* (CYANOBACTERIA) METABOLISM

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ABSTRACT

Cylindrospermopsis raciborskii (Woloszynska) Seenaya & Subba Raju is a freshwater cyanobacterium of worldwide distribution. In the North-eastern region of Brazil many eutrophic water reservoirs are characterized by the dominance of *C. raciborskii*, with recurrent occurrence of blooms. These water bodies have high conductivity due to a high ionic concentration, and are defined as hard (with high values of $CaCO_3$). In this study, we investigated the long-term effect (12 days) of high calcium concentration (8 mM Ca^{2+}) on *C. raciborskii* (T3 strain) growth, morphology, toxin content, and metabolism. Changes in protein expression profiles were investigated by proteomic analysis using 2D gel electrophoresis and mass spectrometry. A continued exposure to calcium had a pronounced effect on *C. raciborskii* (T3): it limited growth, decreased thricome length, increased chlorophyll-*a* content, altered toxin profile (although did not affect PST content, saxitoxin + neosaxitoxin), and inhibited the expression of proteins related to primary metabolism.

Key words: calcium; *Cylindrospermopsis*; paralitic shellfish toxin, proteome.

INTRODUCTION

Cylindrospermopsis raciborskii is a filamentous diazotrophic freshwater cyanobacterium. It is a cosmopolitan species, recognized as highly adaptable to diverse environments (13, 16, 30, 38). The presence of *C. raciborskii* in water supplies used by humans or livestock is potentially harmful since several strains can produce the hepatotoxic alkaloid cylindrospermopsin and other yet unidentified toxins (6, 25). Differently from other geographic regions, several toxic strains isolated from Brazilian water supplies produce paralitic shellfish toxins (PSTs) (19, 25, 26, 36).

C. raciborskii is consistently recorded as a dominant phytoplankton component in eutrophic and hypereutrophic reservoirs of the semi-arid North-eastern region of Brazil (4, 8, 9, 10). In this region, soil composition and climate contribute to the characteristic high ionic composition of water (Na^{2+}, Ca^{2+}, Mg^{2+}, Cl^- and HCO_3^- in millimolar range) yielding

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conductivities up to 25000 μS.cm^{-1} (3). These reservoir waters are also defined as hard, routinely evaluated by calcium carbonate content, with high $CaCO_3$ concentration (> 100 ppm) (11). Water hardness implies high Ca^{2+} concentrations, reported as up to 40 mM in those environments (12).

The influence of salinity (represented by high NaCl concentration) on *C. raciborskii* metabolism has been previously reported and its effect on saxitoxin production has been investigated using a Brazilian strain (T3) (31). However, there is no report on the effect of Ca^{2+} on *C. raciborskii* growth and toxin production. Therefore, considering the common incidence of *C. raciborskii* blooms in water supplies presenting high concentrations of Ca^{2+} (conductivity average 2000 μS.cm^{-1}, max. 19000 μS.cm^{-1} – 3) in this study we verify the effect of a continued exposure to this ion on the physiology of *C. raciborskii* T3 strain, evaluating cellular growth, morphology, chlorophyll-*a*, toxin content and protein expression profiles.

METHOD

Strain maintenance

In this study, the *C. raciborskii* T3 strain was used. This strain was isolated from a toxic cyanobacterial bloom in Brazil (19) and has been maintained in the culture collection of the Laboratory of Cyanobacterial Ecophysiology and Toxicology (UFRJ, Brazil). The T3 strain produces STX, neosaxitoxin (NSTX) and decarbamoyl-NSTX and has been used as a type strain to elucidate the biosynthetic pathway of paralytic shellfish toxins (PSTs) (18, 36). Non-axenic batch cultures of this strain were maintained in ASM-1 medium (15) at 24 ± 2 ºC, with a 12 hour L:D cycle and photon flux of 50 μmol photons.m^{-2}.s^{-1} (provided by common day light fluorescent lamps). Aeration was produced by a compressed air pump. These culture conditions were established according to the environmental conditions from which this strain was isolated.

Preparation of growth medium with added calcium

ASM-1 medium composition has a calcium concentration of 20 μM. In order to simulate a condition where *C. raciborskii* occurs in Brazilian water bodies, $CaCl_2$ was added to obtain 10 mM of the ion calcium. The pH of the culture media was adjusted to 8.0 and the turbidity of both media (ASM-1 and ASM-1 10 mM Ca^{2+}) was measured using a turbidimeter (2100P - HACH). It is expected that part of the added Ca^{2+} will precipitate as $CaCO_3$, which can increase turbidity. Thus, light intensity was measured with a quantum sensor (QST-100 Box – Biospherical Instruments Inc.) immersed in the same volume of culture medium used in the experiments. In order to determine the actual initial concentrations of free Ca^{2+} in ASM-1 and ASM-1 10 mM Ca^{2+}, 10 mL of each medium were analyzed by ion chromatography using a Dionex ISC-1000 system to separate cations. Analysis was performed suppressing conductivity by use of a CS12A column (250 mm, 4 mm internal diameter), preceded by a CG12A column (50 mm, 4 mm internal diameter) and a suppressor pump (CSRS 300 – 4 mm). An isocractic elution with 21 mM metasulfonic acid was performed online using the software Chromeleon 6.80. The sample (ASM-1 or ASM-1 10 mM Ca^{2+}) injection volume was set to 500 μL (loop 500 μL) and the total run time was 15 min. Concentrations were determined considering the retention time and according to a calibration curve of cation standards (Six cation II- Standard, Dionex).

C. raciborskii growth in medium with added calcium

A culture of *C. raciborskii* (T3) strain was maintained in the conditions described above for 12 days. Aliquots of this culture were used as inoculum to experimental tests, which were initiated with 5.0 x 10^5 cells.mL^{-1}. Cells were inoculated into 3-liter glass vessels (flat bottom) with 2 L of ASM-1 medium (control) or ASM-1 with 10 mM high Ca^{2+}, in triplicates. Sampling of each experimental culture condition was done every 3 days during 15 days, under aseptic conditions. Estimation of cellular concentration during growth was performed according to Carneiro et al. (7). Briefly, the length of 30 cells was measured by light microscopy using an

ocular rule. Filaments' lengths were measured on a Fuchs-Rosenthal hemocytometer. The number of cells.mL^{-1} was obtained from the total length of filaments divided by the average cellular length. Growth rates (r_n) were determined according to the following equation:

$$N_t = N_0 \cdot e^{rn \cdot t} \quad [1]$$

Where N_t is the number of cells in time *t*; N_0 is the initial number of cells; and r_n is the growth rate (33).

To measure trichome length, aliquots of the experimental cultures were harvested every 3 days over the course of fifteen days. In each culture triplicate, a minimum of a hundred and fifty filaments were measured using a graduated ocular in a light microscope.

Chlorophyll-*a* measurements

Chlorophyll-*a* (Chl-*a*) content was determined from cells taken every 3 days over the course of 15 days of culturing. Aliquots of 10 mL from each culture were filtered using borosilicate filters (13 mm diameter, Millipore). Cells were transferred to test tubes with 5 mL of methanol (100%) and kept in the dark for 30 minutes. Samples were centrifuged (10000 g, 20 min, 4º C) and the supernatants were used to determine the optical density at 665 nm (with a turbidity correction at 750 nm). Chl-*a* concentration was calculated using Mackiney's extinction coefficient (23), according to the formula:

$$C = [(OD_{665} - OD_{750}) \cdot v] / (V \cdot k \cdot d) \quad [2]$$

where C is Chl-*a* concentration (μg.L^{-1}), v is the volume of methanol (mL), V is the volume of the culture (L), k is Mackiney's extinction coefficient (74.5L·g $^{-1}$·cm $^{-1}$), and d is the distance travelled by light (cm). Chl-*a* is presented as the cell quota (pg.cell^{-1}).

PST analysis

The PST profile of *C. raciborskii* T3 is STX, NSTX and dcNSTX (36). Here, we measured PST production as the sum of STX and NSTX only, since we had no dcNSTX standard available. Culture samples of 500 mL were harvested on the 6th and 12th days and filtered on borosilicate filters (45 mm diameter, Millipore). Cells retained in the filters were analyzed for intracellular PST content while the volume that passed through the filters was analyzed for extracellular PST content. Samples were stored at -20º C until the moment of HPLC analysis, which was performed within 24 h from the extraction procedure. Extraction of toxins was performed using acetic acid according to Carneiro et al. (7). PSTs were analyzed according to Oshima (29) using a Shimadzu HPLC system with a silica-base reversed phase column (125 mm x 4.0 mm, 5 μm; Lichrospher 100 RP 18). The chromatographic condition was: mobile phase – 2 mM heptanesulfonate in 30 mM ammonium phosphate and 6% acetonitrile, pH 7.1. PSTs were detected using a fluorometric detector, with excitation at 330 nm and emission at 390 nm. STX and NSTX were identified and quantified by comparison with known retention time and integrated areas of STX and NSTX standards. The standards were purchased from the Institute of Marine Bioscience – National Research Council of Canada (Halifax, Canada). PST content is presented as cell quota (fg.cell^{-1}).

Protein purification

Samples of soluble proteins were obtained from stationary phase cultures at the twelfth day of culturing. Cells were harvested from a volume of 500 mL by centrifugation (10000 g, 15 min, 4º C) and stored at -20º C. For protein extraction, cells were thawed in ice; 0.5 M acetic acid was added and samples were incubated at 25º C for 1h. An aliquot of 2 mL of the resulting suspension was combined with 18 mL of a solution containing 90% acetone, 10% trichloroacetic acid and 0.07% β-mercaptoethanol, mixed, and maintained at -20º C overnight. The precipitated proteins were recovered by centrifugation (15000 g, 45 min, 4º C) and the resulting pellet was washed three times with 90% acetone. Proteins were

solubilized at 25° C for 1h in a solution of 2 M thiourea, 7 M urea, 4% Chaps, 40 mM DTT, 0.5% Pharmalyte 3-10, 1 mM PMSF. After centrifugation (10000 g, 45 min, 4° C) the supernatant was collected and proteins were further purified using the 2D-Clean up kit (GE Healthcare). The resulting sample had its protein content determined (5) and was stored in liquid nitrogen until use.

2D gel eletrophoresis (2D-PAGE)

Samples containing 150 µg of soluble proteins (in 200 µL of 2 M thiourea, 7 M urea, 4% Chaps, 40 mM DTT, 0.5% Pharmalyte 3-10, 0.002% bromophenol blue) were used to rehydrate strips of 11 cm, pH 4-7 (Immobiline Dry Strip; GE Healthcare) for 16 hours at room temperature. Proteins were focused using the Multiphor II equipment (GE Healthcare) according to the manufacturer recommendations. After focusing, proteins were reduced and alkylated (strips were equilibrated in DTT and iodoacetamide solutions) and the strips were placed on a 12% SDS-PAGE gel (34). For separation of proteins on the second dimension, electrophoresis was carried out on the Multiphor II equipment (GE Healthcare) as a flatbed system, following the conditions recommended by the manufacturer. The gels were stained with Coomassie blue G-250, digitalized, and images were analyzed using the ImageMaster 2D Platinum v5.0. software (GE Healthcare). For each condition tested (control or calcium) three gels were produced, two of them loaded with protein samples extracted from the same culture and a third one with a protein sample extracted from a different culture. Isoelectric point (pI) values of the proteins of interest were determined using a linear 4–7 distribution and the molecular mass (MM) was estimated based on protein low molecular mass markers (GE Healthcare).

Spots on 2D gels were initially detected automatically with ImageMaster 2D Platinum v5.0 software and then manually confirmed. In each gel class (control or calcium) spots intensities were normalized and the three replicate gels were matched (14). A set of spots characteristic of each experimental condition was defined selecting those spots consistently present in replicate gels, thus generating one control master gel and one calcium master gel.

In order to identify differently expressed proteins, images of the two master gels were matched. Spot intensity was evaluated using the relative volume parameter, expressed as:

$$\%Vol = \frac{Vol}{\sum_{S=1}^{n} Vol_s} \times 100 \quad [3]$$

Where, Vol_s is the volume of spot *S* in a gel containing *n* spots.

This is a normalized value which minimizes experimental variations between replicate gels (Image Master 2D Platinum software version 5.0 User Manual). Spots were considered differentially expressed (i) if the mean of $\%Vol$ differed more than two fold between the control and calcium conditions (ii) if the $\%Vol$ values showed little dispersion (mean squared deviation) in replicate gels, and if (iii) dispersion of $\%Vol$ values in each condition did not overlap for more than 25%.

Protein identification by mass spectrometry

Protein spots were cut from gels, washed twice in 50% acetonitrile (ACN) in 25 mM NH_4HCO_3, placed in 100% ACN, and dried under vacuum. Gel fragments were treated with porcine trypsin (15 ng.µl^{-}) (Promega) and peptides were extracted with 50 µL of 50% ACN, 5% trifluoroacetic acid (TFA), vacuum dried, and added to 3 µL of 50% ACN, 0.1% TFA. An aliquot of 0.5 µL of sample was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid matrix in 50% ACN, 0.1% TFA. The mix was spotted onto a MALDI-TOF sample plate and allowed to crystallize at room temperature. MALDI-TOF-TOF analysis was performed in a 4700 Explorer Proteomics Analyzer (Applied Biosystems). Precursor ion fragmentation was obtained using N_2 as collision-induced dissociation gas and collision cell pressure was kept at 2.8×10^{-6} Torr. Trypsin autolysis peptide masses 842.5 and 2211.1 and calibration mixture 1 or 2 (Sequazyme Peptide Mass Standard kit, PerSeptive Biosystems) were used,

respectively, as internal and external standards. The resulting spectra were used to search the NCBI database (version available on 09/06/2010) using the MASCOT program interface (www.matrixscience.com). The search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine and one mis-cleavage of trypsin. A mass tolerance of 100 ppm was used. The criteria for identification were a Mascot score above 50 ($P<0.05$), and a minimum of 2 peptides from the mass spectra with hits in the database. In those cases where identification was provided by a MS/MS spectrum of good quality, the other criteria were not considered.

Statistical analysis

Growth and STX data were expressed as mean values ± standard error (SE). Data for each experimental variable were tested for normality. Differences among standard deviations were determined using Kolmogorov and Smirnov's test and Barlett's test. If data were classified as parametric, ANOVA one-way was performed for multiple comparisons and Tukey-Kramer test to determine the differences between the two treatments. If data were classified as nonparametric, ANOVA was performed using the Kruskal-Wallis (KW) test for multiple comparisons. Dunn´s test was used to determine the specific differences between two treatments. For specific comparisons of STX production, the nonparametric Mann-Whitney U-test was performed. All tests were performed with significance of 95% ($p<0.05$) using the GraphPad Instat 3.0 application. Protein data were analyzed using t-test to compare the relative volume of spots on gel images with significance of 80% ($P<0.2$) using the ImageMaster 2D Platinum software version 5.0 (GE Healthcare).

RESULTS

Measurement of calcium on the culture medium

Measurements of free Ca^{2+} concentrations in the culture media, before inoculation, showed a decreased amount of this ion in relation to the original concentrations, both in ASM-1 (from 20 µM to 17.93 ± 0.01 µM) and in ASM-1 with added Ca^{2+} (from 10 mM to 7.86 ± 0.88 mM). Therefore, cells were maintained in the control condition in 18 µM Ca^{2+} and in high calcium condition in 8 mM Ca^{2+}. Addition of $CaCl_2$ to the culture medium increased turbidity from 3.25 ± 0.38 turbidity units in ASM-1 to 7.28 ± 0.35 turbidity units in ASM-1 added Ca^{2+}.

Effect of calcium on cellular growth, trichome length and chlorophyll-*a*

Cylindrospermopsis raciborskii (T3) presented an exponential growth until the 6th day of culturing with almost the same growth rate both in control ($r_{n\ control} = 0.1972.dia^{-1}$) and high calcium ($r_{n\ calcium} = 0.1809\ dia^{-1}$; $R^2_{control} = 0.9553$ and $R^2_{calcium} = 0.9805$). From that point on, the difference between growth rates in control and calcium became more evident; while control cells showed exponential growth until the 12th day, in high calcium cells apparently started a transition to stationary phase. After 15 days of culturing, cells maintained at 8 mM Ca^{2+} decreased growth rate in comparison to control ($r_{n\ control} = 0.1479.dia^{-1}$ and $r_{n\ calcium} = 0.0827.dia^{-1}$; $R^2_{control} = 0.9362$ and $R^2_{calcium} = 0.8197$) (Fig. 1). In fact, from the 3rd day on, the number of cells was significantly lower in the presence of 8 mM Ca^{2+} as in comparison to control (KW-test, $p<0.01$). Thus, cellular growth was inhibited by a continued exposure to high Ca^{2+}.

The morphology of *C. raciborskii* (T3) grown in these conditions was evaluated measuring trichome length. This trait did not change over the culture period in the control condition (ANOVA, $p>0.05$), but the presence of 8 mM Ca^{2+} resulted in a gradual decrease in trichome length and after 3 days the trichomes were significantly smaller than at control, continuing so during the course of growth (ANOVA, $p<0.01$) (Fig. 2).

While the amount of chlorophyll-*a* ($pg.cell^{-1}$) did not change during the 15 days of culturing in the control condition ($R^2 = 0.0103$), in 8 mM Ca^{2+} cells accumulated more chlorophyll-*a* than in control ($R^2 = 0.7264$), and this difference was apparent from the 3rd day of culturing (KW-test, $p<0.01$) (Fig. 3).

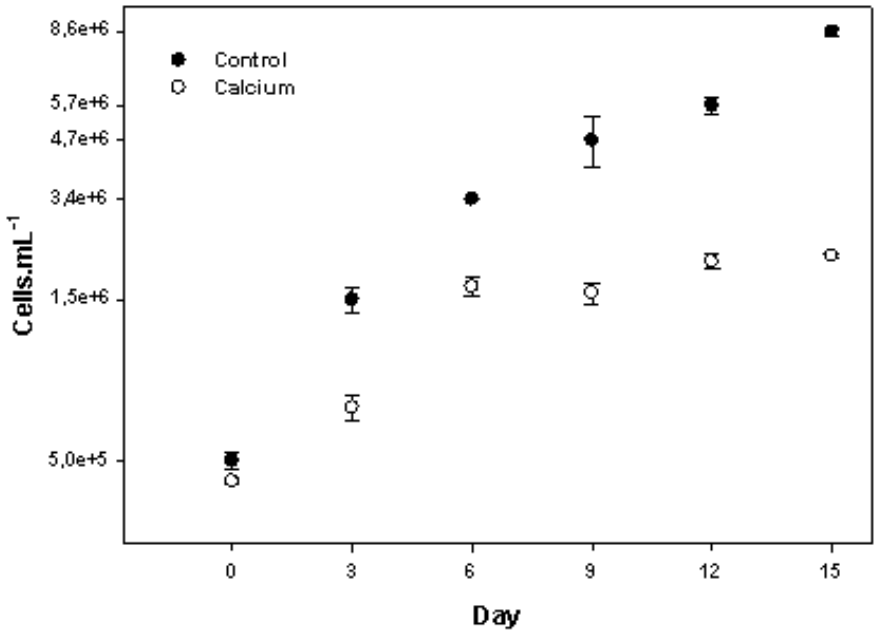

Figure 1. Growth curves of *C. raciborskii* (T3) cells cultured in ASM-1 medium (18 µM Ca^{2+}) or ASM-1 with high calcium (8 mM Ca^{2+}) during 15 days. Cultures were maintained in the following conditions: 24 ± 2 ºC, 12 hour L:D cycle and 50 µmol photons.m^{-2}.s^{-1}, with aeration. Estimation of cellular concentration during growth was done measuring the length of 30 cells by light microscopy on a Fuchs-Rosenthal hemocytometer. The number of cells.mL^{-1} was obtained from the total length of filaments divided by the average cellular length. Error bars are standard errors; n=3.

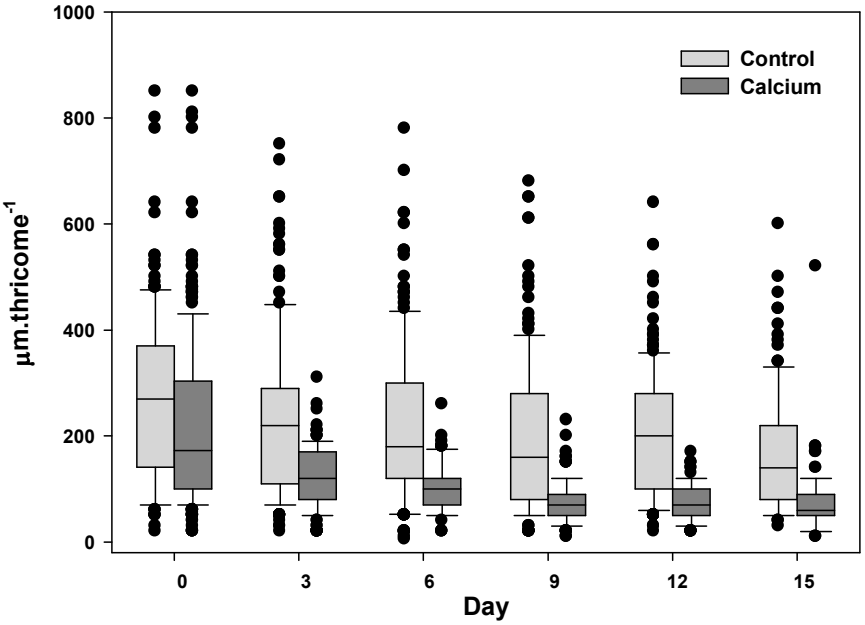

Figure 2. Trichome length of *C. raciborskii* (T3) cells grown in ASM-1 medium (18 µM Ca^{2+}) or ASM-1 with high calcium (8 mM Ca^{2+}) during 15 days. Filaments' lengths were measured on a Fuchs-Rosenthal hemocytometer, using a graduated ocular in a light microscope. Error bars are standard errors; n≥150.

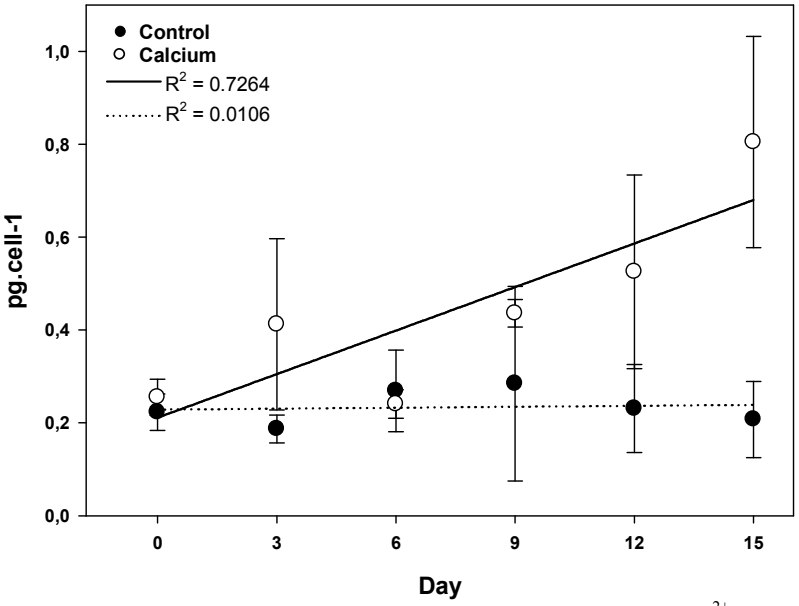

Figure 3. Chlorophyll-*a* content of *C. raciborskii* (T3) cells grown in ASM-1 medium (18 μM Ca^{2+}) or ASM-1 with high calcium (8 mM Ca^{2+}) during 15 days. Chl-*a* was determined from cells taken every 3 days over the course of 15 days of culturing and is shown as the cell quota (pg.cell^{-1}). Error bars are standard errors; n=3.

Effect of calcium on PST content

No difference in intracellular PST content (STX + NSTX) was observed between control and high Ca^{2+} cultures, either at the 6th or 12th day of growth (U-test, P>0.05) (Table 1). Considering only STX, after 12 days at 8 mM Ca^{2+} its cell quota (fg.cell^{-1}) was significantly lower (U-test; P<0.001) than at control, which indicates a conversion of STX into NSTX in the presence of calcium. Extracellular toxin content could not be measured from the culture medium, probably because the values were below the detection limit of the used method (0.3 ng for STX and 0.04 ng for NSTX).

Table 1. PST cellular quota (fg.cell^{-1}) [a] of *C. raciborskii* T3 strain.

Day of sampling	Treatment	STX	NSTX	NSTX+STX	NSTX/STX
6	Control	0.178 ± 0.05	2.211 ± 0.05	2.389 ± 0.56	14.751 ± 4.59
	8 mM Ca^{2+}	0.115 ± 0.04	2.375 ± 0.081	2.490 ± 0.90	21.378 ± 3.15
12	Control	0.978 ± 0.02	4.685 ± 1.80	5.664 ± 2.39	6.316 ± 1.35
	8 mM Ca^{2+}	0.181 ± 0.01[b]	3.086 ± 0.03	3.268 ± 0.03	16.914 ± 0.64[b]

[a] data are presented as the average ± standard error (n=3). [b] significant difference between calcium and control treatments (U-test; p<0.001)

Differently expressed proteins

For the analysis of differently expressed proteins comparing high Ca^{2+} and control condition, 2D gels were done in triplicate for each condition. On average 135 spots were clearly resolved on each gel. Representative 2D gels from both conditions are shown in Figure 4. Considering those spots representing a difference of at least two fold in expression between the two conditions, a total of 35 proteins were defined as differently expressed and selected for identification. From these 35 proteins, only 5 were more abundant in the presence of high Ca^{2+} than in the control condition. Fifteen proteins were identified by MS analysis;

some proteins were identified from multiple spots (spots 33 and 34; spots 63, 64 and 67, Table 2, Fig. 4). These proteins appeared as spots of variable pI values but same MM, possibly resulting from post translational modifications. The remaining proteins could not be identified due to their low amount in the gel spots or to mixture of proteins, resulting in poor quality of mass spectra.

A homology based search in protein databases provided in all cases proteins of cyanobacterial origin as the best results. For each protein, the values of MM and pI estimated from the spot position on the gel and those of the homologous protein retrieved were compared (Table 2). In most cases these values were similar. Putative functions and patterns of differential expression are also presented on Table 2. Most of the proteins were assigned to primary metabolism (energetic metabolism, protein synthesis, photosynthesis). Except for phycocyanin, all the identified proteins were less abundant in high calcium in comparison to control condition.

The most intense signal in all gels corresponded to a group of clustered spots with MM of approximately 20 kDa and pI between 4 and 5, probably containing a few co-migrating proteins. A corresponding gel fragment was processed for peptide identification by MS analysis, which indicated the presence of a phycocyanin subunit (Table 2, Fig. 4).

Table 2. *C. raciborskii* T3 proteins differently expressed in high Ca^{2+}.

Spot no.	Accession	Protein identification (based on homology)	Mascot score	Apparent MM[a] (kDa)/pI[b]	MM[a] (kDa)/ pI[b] of homologue protein	Functional category	Fold change 8 mM Ca^{2+} / control
39	gi|142006	ATPase beta-subunit (*Anabaena* sp.)	97	56/5.10	50.8/5.10	Energy metabolism	10 x less
28	gi|17227501	F0F1 ATP synthase subunit α (*Nostoc* sp. PCC 7120)	164	64/5.1	54.4/5.11		2 x less
124	gi|113475215	Inorganic diphosphatase (*Trichodesmium erythraeum* IMS101)	128	24/4.9	19.3/4.84		2 x less
93	gi|119509590	Pyruvate dehydrogenase E1 β subunit (*Nodularia spumigena* CCY9414)	83	39/5.6	36.4/5.31		10 x less
60	gi|17231623	Phosphoglycerate kinase (*Nostoc* sp. PCC 7120)	81	50/5	42.5/5.15		6 x less
63	gi|17232055	Fructose-1,6-bisphosphate aldolase (*Nostoc* sp. PCC 7120)	127	45/5.3	38.7/5.49		2 x less
64	gi|17232055	Fructose-1,6-bisphosphate aldolase (*Nostoc* sp. PCC 7120)	49	45/5.6	38.7/5.49		2 x less
67	gi|17232055	Fructose-1,6-bisphosphate aldolase (*Nostoc* sp. PCC 7120)	70	45/5.5	38.7/5.49		2 x less
99	gi|186683980	Transketolase, central region (*Nostoc punctiforme* PCC 73102)	59	39/5.6	36.3/5.31		10 x less
119	gi|22299912	Ribulose-phosphate 3-epimerase (*Nostoc* sp. PCC 7120)	52	27/5.9	25.1/5.37	Photosynthesis	2 x less
33	gi|417058	Glutamine synthetase (*Fremyella diplosiphon*)	74	62/5.7	53.5/5.22	Biosynthesis of amino acids	2 x less
34	gi|417058	Glutamine synthetase (*Fremyella diplosiphon*)	60	58/5.6	53.5/5.22		3 x less
65	gi|159154294	Elongation factor Tu (*Palnktothrix agardii* NIVA-CYA 126/8)	78	45/5.0	30.1/4.76	Protein synthesis	2 x less
3	gi|17229234	Molecular chaperone DnaK (Cyanothece sp. PCC 7425)	209	78/4.8	68.0/4.84	Chaperone	2 x less
132	gi|6693856	Superoxide dismutase (*Nostoc commune*)	102	23/5.5	22.3/5.48	Detoxification	3 x less
4	gi|186681644	TonB-dependent receptor (*Nostoc punctiforme* PCC 73102)	63	78/5.6	76/4.68	Transport, membrane receptor	2 x less
115	gi|17232471	Hypothetical protein alr4979 (*Nostoc* sp. PCC 7120)	70	28/5.5	24.2/5.70	Unknown	2 x less
147	gi|20136749	Phycocyanin β subunit (Cylindrospermopsis *raciborskii*)	189	17/6.3	18.4/5.02	Photosynthesis	2 x more

[a]Molecular mass
[b]Isoelectric point

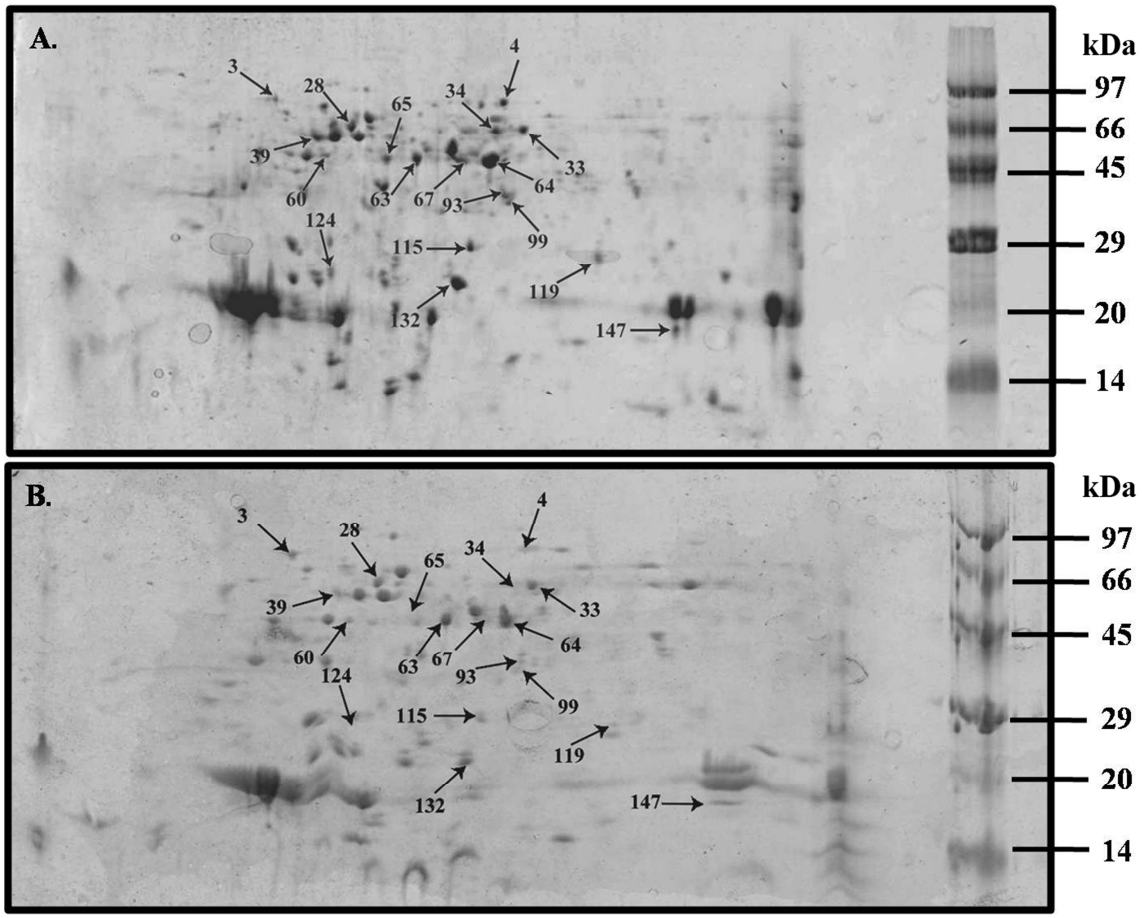

Figure 4. Representative 2D protein patterns of *C. raciborskii* (T3) grown in ASM-1 medium (18 µm Ca^{2+}) (A) or ASM-1 with high calcium (8 mM Ca^{2+}) (B) for 12 days. Soluble proteins were separated using a linear pH 4-7 gradient and a 12% SDS-PAGE; gels were stained with Coomassie G250, digitalized, and compared using an image analysis software. Isoelectric point (pI) values of the proteins were determined using a linear 4–7 distribution and the molecular mass (MM) was estimated based on protein molecular mass markers. Protein spots are identified by the same numbers presented on Table 2.

DISCUSSION

The present work showed for the first time the effect of high Ca^{2+} concentration (8 mM Ca^{2+}) on *C. raciborskii* (T3) metabolism: it limits growth, decreases thricome length and increases cholophyll-a content, alters toxin profile and inhibits the expression of proteins related to primary metabolism. In this study, a concentration of 8 mM defined a high calcium medium, based on the calcium content of some Brazilian water reservoirs in which the dominance of *C. raciborskii* is common (3, 4, 9, 10, 12). This concentration is significantly higher than those reported in other studies with cyanobacteria. For example, 1.25 mM Ca^{2+} for *Lyngbia wollei* (39), up to 0.9 mM for *Microcystis aeruginosa* (37) and 0.2 mM Ca^{2+} for *C. raciborskii* (13) were demonstrated as favourable to growth.

In the present study, upon exposure to high Ca^{2+}, *C. raciborskii* (T3) cells showed a decreased growth rate from the third day of culturing, so a significant decrease in the final number of cells.mL^{-1} after 15 days was observed as compared to control. We also cultivated *C. raciborskii* (T3) in ASM-1

with 5 mM Ca^{2+}, and this condition reduced growth as compared to control but the effect was lower than that observed for 8 mM Ca^{2+}(data not shown). Lower growth in response to salinity was reported for cyanobacteria as an effect of NaCl concentration (up to 40 mM), with inhibition of photosynthetic and respiratory systems (1,2). Moisander et al. (24) showed that the maximum salinity that supported growth of *C. raciborskii* was 70 mM NaCl. Pomati et al. (31) showed that 10 mM NaCl limited growth of the T3 strain after 6 days of exposure and suggested that excess of NaCl impose an energy dispend due to the intense activity of Na^+/H^+ antiporters to maintain cellular homeostasis. Investigations on the tolerance of cyanobacteria to Ca^{2+} have not been reported, except for a recent study showing that for a *M aeruginosa* strain, concentrations from 0.1 to 0.9 mM were suitable for growth (37). It can be postulated that, at high concentrations, similarly to Na^+, the required activity of Ca^{2+} efflux mechanisms would imply an extra energetic demand, which could explain the limited growth rate of cells maintained in the presence of high Ca^{2+} concentrations. In Bacteria, cytosolic free-Ca^{2+} concentrations are controlled by a balance between influx of external calcium via calcium channels and efflux mechanisms such as ATP-requiring Ca^{2+} pumps and Ca^{2+}/H^+ or Ca^{2+}/Na^+ antiporters, so cytosolic free Ca^{2+} above micromolar may be toxic as is the case in eukaryotes (17).

We also showed that thricome length decreased in the presence of high Ca^{2+}. In the studies relating *C. raciborskii* growth and salinity just cited (24, 31), no morphological changes were evaluated. It the case of *M. aeruginosa*, it has been shown that a higher concentration of Ca^{2+}. (0.9mM) induced larger colonial size than a lower (0.1 mM) and increased the buoyancy of the cell population (37).

It has been reported that cultivation of *C. raciborskii* (T3) in the presence of 10 mM NaCl caused accumulation of STX coupled with an increase in cellular Na^+, as compared to control (31). The authors considered the possibility of regulation of STX metabolism in response to cellular Na^+ concentration and suggested that STX biosynthesis can be related to cellular homeostasis. Kellman and Neilan (18) studied *C. raciborskii* saxitoxin biosynthesis *in vitro* and showed that it was inhibited by Ca^{2+} at 1 mM. We observed that, after 12 days, exposure to 8 mM Ca^{2+} resulted in a lower STX content, and consequently higher NSTX. It is possible that the decrease in STX content that we observed in the presence of Ca^{2+} (ca. 5 fold) is a consequence of the conversion among different PST analogues as a result of lower cellular growth and metabolic activity found in this condition, rather than a direct effect of the ion on PST profile. As demonstrated by Soto-Liebe et al. (36), the PST profile of *C. raciborskii* T3 strain is NSTX, decarbamoil-NSTX and STX, in decreasing abundance. Even if we could not measure decarbamoil-NSTX here, still NSTX is the major analogue of *C. raciborskii* T3. Our data show that a high calcium concentration is not sufficient to affect the toxicity of this strain.

The analysis of protein expression profiles comparing control and high Ca^{2+} conditions was performed in order to evaluate the physiological response of the cyanobacterium to the presence of different concentrations of this ion. From the 35 proteins differently expressed, only five were more abundant in high Ca^{2+} in comparison to control. The only identified protein more abundant in high Ca^{2+} than control was a *C. raciborskii* phycocyanin. Phycocyanin is an accessory pigment that participates in the antenna complex, responsible for light harvesting in cyanobacteria (28). In waters with CO_2, carbonate precipitation can occur, so it is expected that a fraction of added Ca^{2+} will precipitate as $CaCO_3$, which is highly insoluble and increases water turbidity (20, 22, 27). Measurements of free Ca^{2+} concentrations in the culture media, before inoculation, indeed showed a decreased amount of this ion in relation to the original concentrations, both in ASM-1 and in ASM-1 with added Ca^{2+}. Addition of $CaCl_2$ to the culture medium also increased turbidity. It can be postulated that the increase in Chl-*a* content that we observed in cells grown in 8 mM Ca^{2+} would be a response to a continuous precipitation of calcium as $CaCO_3$ along the culturing period. The synthesis of pigments would improve light capture by the

antenna complex providing extra energy to compensate for the lower light intensity.

Among the identified proteins less abundant upon exposure to Ca^{2+} as compared to control condition, those involved in energy metabolism represented the majority. The proteins which showed the highest difference in expression between control and Ca^{2+} conditions were ATPase beta subunit, pyruvate dehydrogenase, transketolase and phosphoglycerate kinase. As already mentioned, the decrease in the basal metabolism of *C. raciborskii* in high Ca^{2+} can be due to the extra demand used for the efflux of Ca^{2+}, using transport across the membrane at the expense of ATP. Is must also be considered that at the moment of sampling (12^{th} day of culturing), in the high Ca^{2+} cultures growth rate was significantly decreased. In this case a general lower metabolic activity would be expected in comparison to control (even if some specific enzymes related to Ca^{2+} efflux could be more active). Finally, as already pointed out, measurement of free Ca^{2+} concentrations in the culture media showed a decrease of 10-20% in the amount of this ion in relation to the original concentrations. It is possible that a fraction of the added Ca^{2+} precipitated together with other component such as phosphate, which would also represent a limiting factor for cellular growth (33).

Two proteins with reduced expression in high Ca^{2+} were DnaK and superoxide dismutase. In bacteria superoxide dismutase is constitutively expressed, protecting the cell against oxidative stress, and it is also regulated, increasing as a function of oxygen in stationary phase (35). DnaK is a member of the Hsp70 family of heat shock induced proteins. These proteins are involved in protein folding, secretion and degradation and prevention of protein aggregation. The main signal for the induction of these proteins is a stressful condition, but they are also constitutively expressed in the cell (21). Sod and DnaK were possibly more expressed in control conditions due to the highest growth and metabolic activity of the cells in comparison to cultures exposed to Ca^{2+}.

Another protein less expressed in high Ca^{2+} cultures was a homologue of a TonB dependent receptor. TonB dependent receptors are membrane associated proteins that participate in the active transport of siderophores and nutrients (32). The presence of this protein was not expected in a soluble protein fraction as that used for our analysis and may represent a contamination with the membrane fraction during the procedure of protein isolation. Finally, a protein homologous to a hypothetical protein from *Nostoc* was also less abundant in high Ca^{2+} than in control condition.

The present work indicates that a continued exposure to a high Ca^{2+} concentration is a deleterious condition to *C. raciborskii* (T3) cells, either due to a direct inhibitory effect of an excess of this ion on the cell growth due to the charge to maintain Ca^{2+} homeostais, or indirectly, by changing the chemical composition of the medium. While extrapolation from laboratory conditions to a natural environment should be considered with caution, it can be postulated that in hard waters as those described as natural habitats of *C. raciborskii* in Brazil, high Ca^{2+} concentrations can act as a limiting factor for growth. On the other hand, considering the complex ionic composition of such waters, the presence of other ions may favour bloom formation.

Studies relating limnologic features with composition and temporal dynamics of phytoplankton usually evaluate conductivity, salinity or hardness (3, 12). Such parameters however only poorly define water in terms of specific ionic composition. The work presented here is a preliminary step in investigating, under culture conditions, the effect of different ions individually on the physiology of a toxic *C. raciborskii* strain, which may contribute to identify physicochemical factors that can be related to the dominance and toxigenicity of this species in the environment.

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