

The effects of chloramphenicol, arginine and temperature on PST-production by *Cylindrospermopsis raciborskii* strain D9

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Abstract

The filamentous cyanobacterium *Cylindrospermopsis raciborskii* strain D9, a non-axenic culture isolated from a freshwater reservoir in Brazil, produces PSTs - mainly saxitoxin (STX) and gonyautoxins (GTX2/3), and low amounts of dcSTX and dcGTX2/3. In order to establish conditions to stimulate STX production, we analyzed the effect on growth and toxin production caused by the antibiotic chloramphenicol, arginine and temperature. Quantification was performed by HPLC with post-column derivatization. The toxin profile was confirmed by LC-MS/MS. Chloramphenicol ($1\mu\text{g ml}^{-1}$) inhibits growth and STX and GTX2/3 synthesis after 24 h exposure. Supplementation with arginine (10 mM) diminished cellular STX levels by 78 % after 48 h, while GTX levels were not affected. Growth of this strain was faster at 25 °C than at 19 °C but intracellular PST concentration remained stable until 100 h. The results were substantially different from those previously obtained with *C. raciborskii* strain T3, another non-axenic culture producing STX and the N-sulfocarbamoyl toxins C1-C2. Although the presence of heterotrophic bacteria in both cultures could have an influence on the results obtained, our data suggest that physiological differences between strains are probably related to differential gene expression in the toxin biosynthetic pathways.

Introduction

The paralytic shellfish toxins (PSTs) are a group of tetrahydropurine neurotoxins that selectively block Na^+ channels in excitable cells. Among PSTs, saxitoxin (STX) is the most potent analogue. The toxins are produced by representatives of widely divergent phyla, primarily by marine dinoflagellates and cyanobacteria from marine and freshwater ecosystems throughout the world (Llewellyn 2006). *Cylindrospermopsis raciborskii* is a freshwater planktonic cyanobacterium, comprising strains that can produce cylindrospermopsin (CYL), a potent hepatotoxic alkaloid, or PSTs, but many do not produce either toxin. These filamentous strains are always maintained as non-axenic cultures, i.e. accompanied by heterotrophic bacteria. The biosynthetic pathways involved in CYL production are well defined, but the genetics of PST synthesis in cyanobacteria and dinoflagellates are not yet elucidated and regulatory factors remain unknown.

Our aim was to characterize the PST production of *C. raciborskii* strain D9 under different culture

conditions that might induce or suppress toxin biosynthesis. We supplemented cultures with arginine, one of the substrates putatively involved in STX synthesis. Toxin biosynthesis and cell toxin content was also evaluated in the presence of the antibiotic chloramphenicol (CAM). This reversible inhibitor of ribosomal protein synthesis was previously shown to affect PST production in *C. raciborskii* strain T3, also isolated from a freshwater habitat in Brazil (Pomati *et al.* 2004). Finally, we determined the effect of temperature on growth and toxin production in strain D9, as an external factor potentially affecting toxin biosynthesis.

Methods

Cyanobacterial cultures and treatments

Cylindrospermopsis raciborskii strain D9 was clonally isolated from a mixed culture SPC338 kindly provided by María Teresa de Paiva from the Billings water reservoir near Sao Paulo, Brazil. It was maintained in 250-ml glass flasks with MLA medium (Castro 2004) at 18-23 °C under a 12:12 h L:D photoperiod with

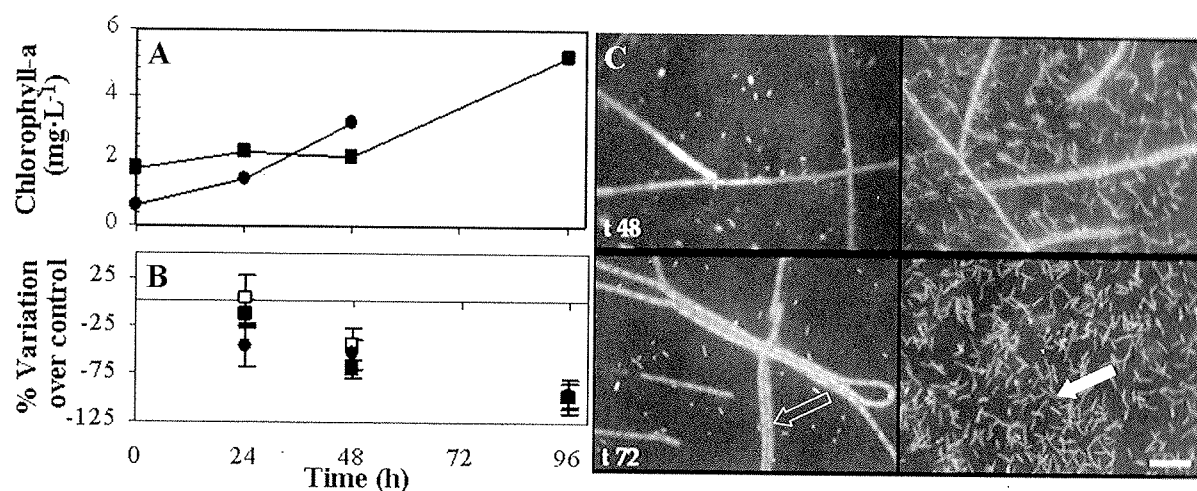


Figure 1. Effect of arginine on growth (A) in cultures with culture medium MLA (■) and arginine 10mM added (●) and in intracellular toxin levels (B) of GTX 3 (■) GTX 2 (□) and STX (●) in cultures of *C. raciborskii* D9. Data points represent the percentage mean variations over control ($\pm$ standard error). In (C) the left panel shows cyanobacteria (open arrow) and heterotrophic bacteria (closed arrow) cultured in MLA; the right panel corresponds to cultures with arginine added. Scale bar=10 μ m.

33 μ mol photons $m^{-2} s^{-1}$. To evaluate effects of CAM and arginine on toxin production, cultures of *C. raciborskii* strain D9 were exposed to CAM (1 μ g ml^{-1}) and sampled for toxin analysis at 0, 8 and 24 h after exposure. We first determined the CAM concentration and the range for which no measurable effects on chlorophyll *a* synthesis were observed. These conditions were: 1 μ g ml^{-1} CAM and between 0 and 8 h culture (initial chlorophyll *a* concentration 0.3 mg L^{-1}). Cultures exposed to arginine (10 mM final concentration) were harvested at 24, 48 and 96 h for toxin quantification. Temperature effects on toxin production were evaluated in cultures grown at 19 and 25 $^{\circ}C$ under continuous white light at 114 μ mol photons $m^{-2} s^{-1}$. Growth of cultures was estimated by chlorophyll quantification. Cell integrity was evaluated by epifluorescence microscopy in samples stained with acridine orange (Hobbie 1977).

Toxin preparation and analysis

One ml of cyanobacterial culture was harvested by centrifugation at 16000 $\times$ g for 15 min and the cell pellet was lyophilized. PSTs were extracted in 300 μ l of 0.05M acetic acid by bath-sonicating twice for 30 min, with 1 h of soaking between sonications; samples were then centrifuged at 5220 $\times$ g for 10 min. The supernatant was filtered through a 0.45- μ m pore size membrane filter and stored at -20 $^{\circ}C$ until analysis. In addition, samples of the culture medium after growth (but minus cells) were harvested for analysis of extracellular toxins.

Initial quantitative analysis by high performance liquid chromatography with fluorescence detection (HPLC-FLD) was carried out according to the method described in Lagos (1999), using a Jasco HPLC chromatograph PU-2089 coupled with Jasco FP2020Plus fluorescence detector, with some minor modifications. Toxin analysis was performed in reverse-phase on a Kromasil C-8 column (100 $\times$ 4.6 mm i.d.), with oxidant and acidifying reagent flow rates at 0.7 $ml min^{-1}$.

Mass spectral measurements were carried out on a triple quadrupole mass spectrometer (API 4000 QTrap, ABI-Sciex) with turbo-spray ionization in negative ion mode. The analytical column was a Hypersil BDS C8 (50 $\times$ 2 mm, 3 μ m, 120 $\text{\AA}$), with a flow rate of 0.3 $ml min^{-1}$ using a binary elution gradient (A: water and B: 95 % acetonitrile/methanol (1:2 v/v) and 5 % water. Both eluants contained 2.0 mM ammonium formate and 50 mM formic acid. Initial composition was 40 % Eluant B with a linear gradient to 100 % B at 6 min, isocratic 100 % B until 15 min, then returning to initial conditions.

Toxin identity was confirmed by comparison of mass spectral data from sample peaks with external standards of certified reference PST obtained from the CRMP programme from NRC, Halifax, Canada.

Results

The LC-MS/MS analysis showed that *C. raciborskii* strain D9 produces the following PST profile: STX, GTX2/3, dcSTX, and dcGTX2/3 (Table 1). The main toxins detected in both intracellular and extracellular fractions were STX and GTX2/3. Neosaxitoxin

(NEO) was only detected in the extracellular fraction.

The experiments performed under the specified antibiotic conditions showed that CAM increased STX cell content (90 % of variation over control), but not the intracellular concentration of GTX2/3 (data not shown). After 24 h in culture, total cell lysis was observed.

Table 1. Determination of PSTs by LC-MS/MS in *C. raciborskii* D9.

	Extracellular (ng·µl ⁻¹)	Intracellular (ng·mg ⁻¹)
STX	0.131	59.2
NEO	0.021	nd*
dcSTX	0.004	2.9
GTX2/3	1.369	1,242
dcGTX2/3	0.074	8.9

* Not detected

Arginine at 10 mM concentration did not affect growth until 48 h of exposure in culture (Fig. 1A), but mean intracellular STX levels nevertheless decreased 48 % relative to controls after 24 h. GTX2/3 levels were not affected during this exposure (Fig. 1B). The amount of heterotrophic bacteria in the medium increased in cultures supplemented with arginine, with concomitant cyanobacterial cell lysis (Fig. 1C).

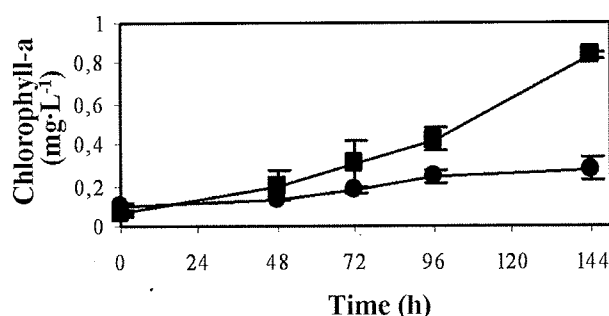


Figure 2. Effect of the temperature on growth at 19 °C (●) and 25 °C (■) of *C. raciborskii* D9.

Growth of *C. raciborskii* D9 was four-fold higher at 25 °C than at 19 °C by 144 h as determined by chlorophyll-specific biomass measurements (Fig. 2). However, intracellular toxin content, standardized by dry weight, remained stable in that period. No changes were observed in the morphology of filaments under growth at the different temperatures.

Discussion

The toxin profile of *C. raciborskii* strain D9 (STX, dcSTX, GTX2/3 and dcGTX2/3) is radically different from that of the previously described strain T3 (STX, C1 and C2) (Pomati *et al.* 2004). The PST profile differences indicate functional modifications of the basic PST biosynthetic pathway, related to the expression and activity of carbamoylase in strain D9, versus putative N-aminosulfotransferase activity in strain T3. These differences are genetically determined and intracellular processes, unrelated to the presence of heterotrophic bacteria. Nevertheless, detection and confirmation by mass spectral analysis of NEO in the extracellular medium indicates that biotransformation (possibly mediated with heterotrophic bacteria) also occurs in cultures of strain D9.

Among other differences between *C. raciborskii* strain D9 and the homologous strain T3, CAM treatment differentially affected STX production. We observed an increase in STX cell content in strain D9 at CAM concentration of 1 µg ml⁻¹. This may be an indication of greater antibiotic sensitivity of strain D9, with the rate of STX biosynthesis uncoupled from the declining growth rate of the cells and eventual lysis. Nevertheless, it is also possible that the negative effect on growth rate of heterotrophic bacteria could indirectly influence the cyanobacterial response. The presence of high arginine in the medium also had a different effect on strain D9 than on T3. In D9, the 48 % decrease in cell STX quota after 24 h exposure to arginine can be compared to the reported 476 % increase in strain T3 (Pomati 2004). Arginine is a putative precursor of STX, and therefore it is reasonable to expect an induction of STX production, and an increase in cell quota unless the growth rate is proportionally increased (apparently not the case). In strain D9, when arginine was supplemented, growth of heterotrophic bacteria in the cultures was greatly induced, presumably as a response to provision of a heterotrophic growth substrate. Although at 24 h in culture this effect was not dramatic, we cannot discard the possibility that negative allelochemical interactions between cyanobacteria and heterotrophic bacteria may have been stimulated.

We previously described that over a long period in culture (50 days), temperature had an effect on PST production in strain C10 (Castro 2004). However, in D9 cultures, we did not detect temperature-dependent inhibition or stimulation of PST production over 144 h. For cylindrospermopsin-producing strains, an in-

crease in toxin cell quota was observed when the cultures were grown below optimal temperatures (Saker and Griffiths 2000). However, that was not the case for PST in the D9 strain. High genotypic variation among strains, in growth, metabolism and biosynthetic rates argues strongly for caution in forming general conclusions on regulation of toxin synthesis by environmental factors.

Acknowledgements

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References

- Lewellyn, L. (2006). *Nat. Proc. Rep.* 23: 200-222.
- Pomati, F., Moffitt, M.C., Cavaliere, R. & Neilan, B.A. (2004). *Biochim. Biophys. Acta* 1674: 60-67.
- Castro, D., Vera, D., Lagos, N., García, C. & Vásquez, M. (2004). *Toxicon* 44: 483-489.
- Hobbie, J.E., Daley, R.J. & Jasper, S. (1977). *Appl. Environ. Microbiol.* 33: 1225-1228.
- Lagos, N., Onodera, H., Zagatto, P., Andrinolo, D., Azevedo, S. & Oshima, Y. (1999). *Toxicon* 37: 1359-1373.
- Saker, M.L. & Griffiths, D.J. (2000). *Mar. Freshwater Res.* 52: 907-915.