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Calcium-Boron Interaction in Exopolysaccharide Production by the Cyanobacterium, *Nostoc spongiaeforme*

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Abstract The effect and interaction of Ca and B on exopolysaccharide (EPS) synthesis in the diazotrophically growing cyanobacterium, *Nostoc spongiaeforme*, was investigated. The absence of B inhibited EPS synthesis 1.56-fold (16 μg glucose equivalent/mg dry weight, 16 d) over the control cells (25 μg glucose equivalent) grown in medium containing 0.5 mM Ca and 8 μM B. When B concentration was raised to 40 μM, EPS production was stimulated 1.8-fold. Reduction of Ca concentration to one-half (0.25 mM) resulted in increased B demand (16 μM) by the cells for EPS production at par with the normal sets. However, without Ca, EPS production also increased as B increased. Addition of B to a Ca-free medium stimulated cyanobacterial diazotrophic growth as well as synthesis of Chl *a* and phycocyanin (0–8 d). The data suggest B-dependent diazotrophic growth during Ca-deficiency and point to an important interactive role of Ca and B in regulation of cyanobacterial EPS synthesis.

Key words: *Nostoc*, exopolysaccharide, boron, calcium

In cyanobacteria (e.g. *Anabaena cylindrica* [2]), EPS production is regulated by the C:N ratio. Such sulfated polysaccharides are acidic having galacturonic acid along with glucose and mannose as the main sugar component [16, 17, 18]. Cyanobacterial exopolymers compare well with those extracted from bacteria and archaea and, therefore, offer an ideal resource material for large-scale deployment in biotechnological and biopharmaceutical applications [25, 6]. Calcium is an important ingredient of most culture media and cyanobacterial slime contain as much as 5.25 mg Ca/g [17]. It is also involved in calmodulin-mediated regulation of cellular processes [12], and has been implicated in regulation of heterocyst frequency and nitrogenase activity [22]. Ca-deficiency lowers photopigment content in cyanobacteria whose photosynthesis and nitrogen fixation are accompanied by increased B demand, and possibly the uptake and/or binding of Ca may be regulated by B [4]. B requirement is specific to diazotrophic growth; B is apparently not required by cells growing on combined nitrogen [14]. Ca stabilizes glycolipid in the inner layer of heterocyst to restrict O_2 diffusion [9]. It also protects nitrogenase from O_2-inactivation in non-heterocystous forms [7]. Recent studies have revealed a structural role of Ca^{2+} in cell wall and cell membrane of prokaryotes [20].

The present communication describes the role of Ca and B and their interaction in respect to EPS synthesis, pigment production, and general growth in the diazotrophic cyanobacterium, *Nostoc spongiaeforme*.

Materials and Methods

Organism and Growth Conditions

Nostoc spongiaeforme Agardh ex Born. et Flah. was grown diazotrophically in modified Chu-10 medium [10] lacking any combined nitrogen source, under the cool white fluorescent illumination (14.4 W/m^2) with 18:6 h light/dark cycles at 24±1°C. The nutrient solution contained macroelements (g/l): $MgSO_4 \cdot 7H_2O$, 0.025; Na_2CO_3, 0.020; $Na_2SiO_3 \cdot 5H_2O$, 0.044; $CaCl_2 \cdot 2H_2O$, 0.0735; K_2HPO_4, 0.01; Fe-citrate 0.0035; citric acid 0.0035, and microelements (mg/l): H_3BO_3, 0.50; $ZnSO_4 \cdot 7H_2O$, 0.05; $MnCl_2 \cdot 4H_2O$, 0.05; $CuSO_4 \cdot 5H_2O$, 0.02; MoO_3, 0.01; and $CoCl_2$, 0.04. All the chemicals were laboratory grade products of Glaxo India Ltd., Mumbai, India.

Cells were washed 6 times in 10 mM EDTA followed by deionized water prior to their growth in either full-strength Ca (0.5 mM of $CaCl_2 \cdot 2H_2O$), or half-strength Ca (0.25 mM), or in Ca-free medium. For Ca-B interaction experiments, the basal B level (8 μM as H_3BO_3) was varied as 16, 32, 40, or 80 μM along with the sets lacking this salt. The parameters compared were EPS production in long-term (0–16 d), and general growth and pigment biosynthesis in short-term (0–8 d) experiments.

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Protein Estimation

Protein was estimated by the method of Lowry *et al.* [13] as modified by Herbert *et al.* [11] using bovine serum albumin (Sigma) as standard.

Dry Weight Determination

Cells were collected by centrifugation, washed, and dried (60°C) to constant weight (expressed as g/l).

Extraction and Determination of Pigments

A known volume of cyanobacterial culture (10 ml) was taken out at predetermined intervals, centrifuged (3,000 rpm), and the pellet suspended in 10 ml acetone (80%) for overnight incubation in the dark (4°C). The supernatant was used to measure Chl *a* (665 nm) in a Bausch and Lomb Spectronic-20 colorimeter. Phycocyanin from the remaining blue residue was extracted in deionized water through 2–3 cycles of freezing and thawing, and the absorbance was taken at 620 nm. Pigment concentrations (mg/g biomass dry weight) are based on absorbance coefficients of 82.04 (Chl *a*, [15]) and 7.5 (phycocyanin, [5]), respectively, according to the equation $\propto = D/dc$ where D = optical density of the pigment, d = inside pathlength of the cuvette (cm) and c = pigment concentration, g/l.

Exopolysaccharide Extraction and Estimation

EPS was extracted by the procedure reported earlier [21]. Cells were centrifuged at room temperature (10,000 ×g, 5 min) to collect the biomass pellet for separation of the associated EPS. The resulting supernatant was concentrated (10-fold) by evaporation (40°C) for isopropanol precipitation. The pelleted biomass suspension in appropriate volume of double-distilled water was stirred gently followed by centrifugation at 4°C (10,000 ×g, 10 min). The EPS released was mixed 1:1 with chilled isopropanol for precipitation and the precipitate washed (3–4 times) with isopropanol (50%, v/v) to remove the adhering salts in the medium. The EPS extract was oven dried (37°C) and acid hydrolyzed (HCl, 2 M) at 100°C (2 h) as suggested by Panoff *et al.* [16]. Appropriately diluted hydrolysate (in distilled water) was analyzed for glucose (μg) by the method of Dubois *et al.* [8]. Total EPS corresponded to the amount liberated after cold centrifugation of cyanobacterial cells plus that already liberated into the ambient growth medium. EPS concentration is expressed as glucose equivalent/mg biomass dry weight.

All experiments were carried out in triplicate with standard errors represented as bars wherever necessary.

Results and Discussion

B is essential for N_2-dependent growth of cyanobacteria [14]. The notable consequence of B-deficiency in cyanobacterial cells is decline in contents of phycobiliprotein and Chl *a*,

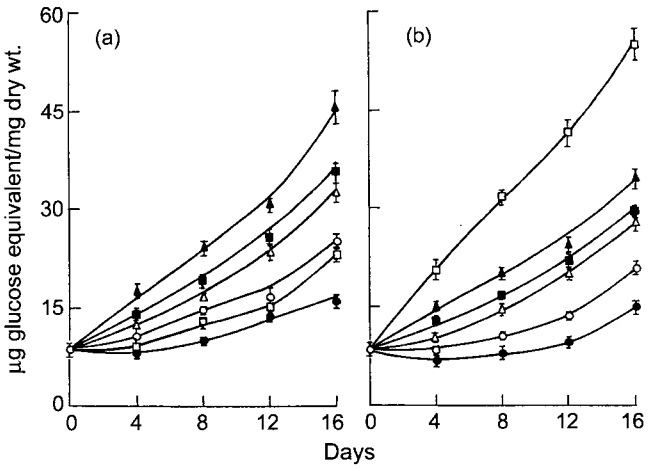

Fig. 1. Exopolysaccharide production by *N. spongiaeforme*. (a) Normal Ca (0.5 mM) and varying B concentrations as control 8 μM B (○–○), 16 μM B (△–△), 32 μM B (■–■), 40 μM B (▲–▲), 80 μM B (□–□), and those lacking B (●–●). (b) Half-strength calcium (0.25 mM) and varying B concentrations as control 8 μM B (○–○), 16 μM B (△–△), 32 μM B (■–■), 40 μM B (▲–▲), 80 μM B (□–□), and those lacking B (●–●).

accumulation of carbohydrates, and yellowing and breakage of cells. Mateo *et al.* [14] implicated B directly in nitrogen fixation because the element facilitates carbohydrate transport from vegetative cells to heterocyst. In the diatom *Cylindrotheca fusiformis* (which does not fix nitrogen), B-deficiency increased carbohydrate content as a consequence of enhanced photosynthetic activity [23] and reduced ion uptake [24].

We have monitored EPS synthesis during diazotrophic growth of *Nostoc spongiaeforme* as regulated by B and the possible mode of B-Ca interaction. The data in Fig. 1a show the course of EPS production at varying B concentrations (8–80 μM) while keeping Ca concentration constant at 0.5 mM (the optimum concentration for growth of the cyanobacterium). EPS production in control cells (8 μM B) attained a maximum of 25 μg glucose equivalent/mg dry wt (on the 16th day) while B-deficiency caused a drastic decline as seen from the slower pace and the lowest yield (16 μg glucose equivalent). The stimulation of EPS production by the cells grown in a 2-fold higher B level (16 μM) could be discerned at the 4th day and ultimately reached 1.3-fold (32.5 μg glucose equivalent) over the normal B set. Therefore, it appears that, at least for EPS synthesis, the prescribed B level (8 μM) in the growth medium was sub-optimal. The stimulation of EPS production in 32 μM B was only 1.1-fold. Still higher B level (40 μM) stimulated EPS synthesis to a maximum of 45.42 μg glucose equivalent. We grew *Nostoc* in 40 μM B rather than the one (30 μM) used by Bonilla *et al.* [4] for *Synechococcus* PCC 7942, where a higher B level (400 μM) with Ca (0.5 mM) comparable to the present study proved to be inhibitory for growth and Chl *a* synthesis in *N. spongiaeforme*. Still higher B concentration (80 μM) inhibited EPS synthesis in

the test cyanobacterium as it ultimately reached an all-time low of 23 μg glucose equivalent. The overall trend indicated that B regulation of EPS synthesis in the test cyanobacterium was almost similar to that of nitrogenase activity or pigment synthesis as reported in *Anabaena* sp. PCC 7119 [14], and also in some other heterocystous and non-heterocystous cyanobacteria [3].

Bonilla *et al.* [4] reported that, unlike in Ca-deficient cells of *Synechococcus* PCC 7942, B-deficency (with normal Ca strength) increased general growth (dry weight and chlorophyll) of this strain in long-term experiments; in other words, Ca-deficiency was relieved by addition of B. Interestingly, the growth inhibitory B level (400 μM) in full-strength Ca (0.5 mM) was rendered less toxic when Ca level was reduced to half. Our result on EPS production by *N. spongiaeforme* has revealed an increased B demand by the cells grown in half the concentration of Ca level (0.25 mM, Fig. 1b). For example, the otherwise inhibitory B level (80 μM) under conditions of full-strength Ca, proved to be stimulatory (55 μg glucose equivalent) when the Ca content was reduced to half (0.25 mM). The increased B demand by *N. spongiaeforme* cells under Ca-deficiency became apparent as the basal B level (8 μM) could support EPS synthesis to a maximum of only 21 μg glucose equivalent. Such a 'Ca-effect' could be counteracted only marginally in enhanced B level of 16 μM (27.94 μg glucose equivalent); the contribution of 32 μM B was slightly high in this regard (29 μg glucose equivalent). It is interesting to note that a still higher B level (40 μM), that supported maximum EPS production for full-strength Ca sets, was rendered less effective as the yield was limited to 34.5 μg glucose equivalent, probably due to Ca-deficiency. According to Bonilla *et al.* [4], *Anabaena* and *Synechococcus* recovered well from high B toxicity in terms of general growth, pigment biosynthesis, and even partial recovery of thylakoid structure under conditions of Ca-depletion, thus indicating that B supply may be crucial only during Ca-limitation. A reciprocal interaction between Ca and B is conceivable in such a case. It was proposed that B might facilitate the uptake of Ca in a manner indicating B requirement for growth under conditions of low Ca and both the elements were suggested to interact through their cellular binding sites, and, possibly, the binding of B could facilitate or even enhance that of Ca [4].

Our experiments on EPS production by *N. spongiaeforme* in Ca-free media supplemented with different concentrations of B (16–80 μM) indicate that with normal B (8 μM), EPS production is suppressed 12.37-fold and can be relieved only partially by 16, 32, or even optimally by 40 μM of B to reach a 23.75 μg glucose equivalent or even very close to normal Ca and B control by 80 μM (24.73 μg glucose, 16d; Table 1). The overall trend reflects the essentiality of Ca for EPS synthesis in the test cyanobacterium and points to the critical role of B when Ca is either low or absent in the ambient environment.

Table 1. Exopolysaccharide production in *N. spongiaeforme* grown as control and in Ca-lacking sets containing varying B levels (16th day).

	Boron concentration in growth medium (μM)	Exopolysaccharide (μg glucose eq/ mg dry wt) (±S.E.)
Control (0.5 mM Ca)	8	25±1.25
Experimental (minus calcium)	8	2.02±0.090
	16	10.8±0.324
	32	14.0±0.56
	40	23.75±1.18
	80	24.73±0.86

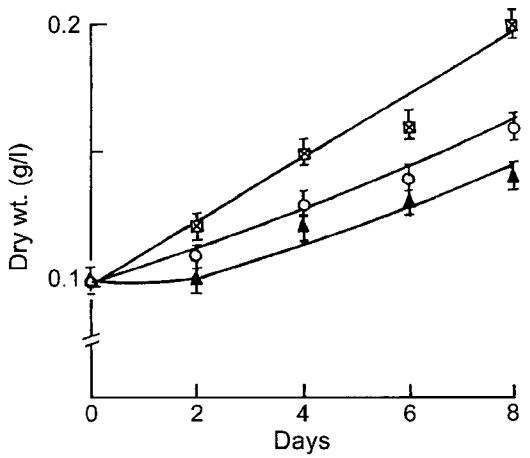

Fig. 2. Diazotrophic growth of *N. spongiaeforme* cells (0–8 d) as control (0.5 mM Ca + 8 μM B) (□–□), 0.25 mM Ca + 8 μM B (×–×), minus Ca + 8 μM B (▲–▲), and minus Ca + 80 μM B (○–○).

Our data (Fig. 2) also support the earlier reports on Ca requirement for various cellular processes in cyanobacteria [17, 12, 22] as *N. spongiaeforme* biomass reached a maximum of 0.2 g/l within 8 days in controls (normal Ca and B), but the diazotrophic growth remained mostly unaffected when the Ca level was reduced to half. The growth yield of the organism can be reduced by 1.4-fold (0.14 g/l) due to lack of Ca although having normal B, and this depression in growth could be recovered only 1.1-fold by a 10-fold higher B supply (80 μM). The data point to a higher B demand by cells recovering from a total lack of Ca in terms of growth as applicable to EPS synthesis; however, in no case, the recovery was 100%. The lowering of diazotrophic growth of the test cyanobacterium (–Ca + 80 μM B) may perhaps be a reflection of N-starvation caused by the lack of Ca and the possible substitution of the element by B, as reported earlier [4].

Short-term experiments for monitoring of Chl *a* indicated that the reduction of Ca levels to 50% had an adverse

Table 2. Chlorophyll *a* and phycocyanin production in *N. spongiaeforme* in control, in half-strength Ca, and in Ca-lacking sets with normal and high B concentration (80 μM) (mg/g dry wt).

		0 day	2nd day	4th day	6th day	8th day
Control (0.5 mM Ca plus 8 μM B)	Chl.*a*	4.87±0.146	8.12±0.28	9.57±0.287	9.63±0.385	11.44±0.343
	PC	54±1.89	89.17±3.57	105±3.15	116.87±4.09	126.36±5.05
0.25 mM Ca plus 8 μM B	Chl.*a*	4.87±0.146	6.09±0.183	7.27±0.291	8.71±0.261	9.87±0.345
	PC	54±1.89	75±2.25	90±3.6	100±3.5	126.29±5.08
Minus Ca plus 8 μM B	Chl.*a*	4.87±0.146	1.71±0.0769	3.57±0.143	3.60±0.144	6.81±0.20
	PC	54±1.89	24.54±0.858	51±1.53	92.31±3.69	43.75±1.53
Minus Ca plus 80 μM B	Chl.*a*	4.87±0.146	2.21±0.066	5.28±0.211	7.28±0.218	8.37±0.29
	PC	54±1.89	32±0.96	80±2.8	96.25±2.88	56.5±2.26

effect on pigment biosynthesis in contrast to the general growth just described (Table 2). This may be because the pigment level showed a marked lowering even on the second day, and the same trend continued until the end of the experiment, amounting to an approx. 1.16-fold lowering (9.87 mg Chl *a*/g dry wt) from that in normal cells (11.44 mg). General growth in Ca-free sets started after a lag of 2 days (Fig. 2), whereas Chl *a* synthesis initially fell 2.85-fold in the same period. A slow recovery led to just 6.81 mg Chl *a*, thus amounting to an approx. 1.7-fold lowering compared to normal cells. Whereas the cells resumed normal growth in 80 μM B (Fig. 2), Chl *a* biosynthesis was invariably depressed initially and took about 4 days to reach just 8.37 mg, a value still lower (1.36-fold) than the normal set. Subsequent follow-up of PC synthesis under similar conditions indicated that control cells of *N. spongiaeforme* increased their PC level to 126.36 mg in the 8th day (Table 2). The mode of PC biosynthesis for the one-half Ca level sets was generally similar to that for Chl *a* described, with the exception that the cells eventually recovered fully as seen from the value of 126.29 mg, which is nearly similar to that in controls. A total lack of Ca (but having normal B) was detrimental to PC synthesis as evident from the abrupt 3.6-fold decline (24.54 mg/g) over the control during the 2nd day. Further incubations upto the 6th day, however, apparently favoured PC biosynthesis, whose level increased to 92.31 mg. The pigment level declined again to nearly 50% (43.75 mg) on the 8th day. Similar sets (Ca-lacking) to which 80 μM of B was added responded positively in terms of Chl *a* synthesis (Table 2) and PC synthesis depended on the presence of Ca after the 6th day, irrespective of the high B dose (80 μM), as the pigment level ultimately declined to 56.5 mg. Such a high sensitivity of PC towards Ca-depletion may be attributed to the initial N-starvation in *N. spongiaeforme*, possibly through depression of nitrogen fixation [19] and the resultant consumption of the phycobilin as a nitrogen source [1]. It also appears that after 6 days of incubation, the cells may have become N-limited once again, and hence resorted to phycobilin consumption.

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