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Nutrient Stress Causes Akinete Differentiation in Cyanobacterium *Anabaena torulosa* with Concomitant Increase in Nitrogen Reserve Substances

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ABSTRACT. Addition of nitrogen source (nitrate), carbon sources (acetate, citrate and fructose), depletion of nutrients (phosphate-free nitrate medium), dilution of medium (2, 4 and 8 times diluted nitrate medium) under unaerated conditions induced akinete differentiation in *Anabaena torulosa*. Aerated cultures under the same conditions did not differentiate akinetes. The amounts of reserve metabolites – glycogen and cyanophycin (multi-L-arginyl-poly-L-aspartic acid) granule polypeptide (CGP) – were determined in unaerated and aerated cultures, and at different stages of growth and akinete differentiation. The addition of nitrate, acetate, citrate and fructose under unaerated conditions resulted in the accumulation of glycogen and CGP in higher amounts after 4 d (akinete initiation); the CGP content further changed at mature free akinetes phase. Higher accumulation of reserve products was also observed under nutrient deficiency (phosphate-depleted or diluted media) after 4 d of cultivation. Under aerated conditions reserve product accumulation was considerably lower. Thus a low accumulation of reserve products in aerated cultures showed that aeration probably somehow relieves the organism from a nutritional stress.

Cyanobacteria developed a diversity of adaptive mechanisms for survival in extremes of environmental, nutritional and other stresses (Hagemann and Erdmann 1977; Brown 1990; Potts 1996; Joset *et al*. 1998; Agrawal and Misra 2002; Agrawal and Singh 2002; Agrawal and Pal 2003). Under conditions of imbalanced nutrition, cyanobacteria synthesize reserve compounds (Ascencio *et al.* 2003), often designated as inclusion bodies (Allen 1984). Of these glycogen (Glg) and cyanophycin (multi-L-arginyl-poly-L-aspartic acid) granule polypeptide (CGP) constitute the carbon and nitrogen reserves, respectively. CGP accumulation is observed under nutrient limitation with phosphate and sulfate; under certain environmental stress, such as low light intensity, extreme temperature (Allen 1984) and exposure to toxic metals, *e.g.*, cadmium (Bonilla 1995). Accumulation of Glg and CGP in response to salt stress and their decrease upon relieving the salt stress in the cells of *Scytonema* sp. has been reported. Although accumulation of Glg and CGP during akinete differentiation in *Anabaena torulosa* has been reported (Sarma and Swarn Kanta 1979, 1980; Swarn Kanta and Sarma 1980; Sarma and Khattar 1986*a*,*b*) it would be interesting to know which of these two reserves is conserved in higher amounts during akinete formation.

Our previous studies identified aeration as a new environmental factor that regulates akinete differentiation in *Anabaena torulosa* (Sarma *et al*. 1998, 2000); we therefore investigated the accumulation of Glg and CGP in response to nutritional stress in unaerated (akinete forming) and aerated (non-akinete forming) cultures.

MATERIALS AND METHODS

Organism and culture conditions. *Anabaena torulosa* (CARM) LAGERH ex BORN et FLAH, an isolate of our laboratory (Sarma and Swarn Kanta 1979), was propagated axenically in a nitrate-free medium (Allen and Arnon 1955) (without any addition of nitrogen, designated here as N_2 medium) in a culture room at 28 ± 2 °C and illuminated with daylight fluorescent tubes for 14 h per d, giving a radiant flux of 9.5 W/m^2. Such cultures of the organism represent unaerated controls which were shaken by hand thrice daily; separate batch cultures were cultivated with bubbling of *Millipore* membrane-filtered air (O_2–N_2; 21 : 79, *V*/*V*) at a flow rate of 10 mL/h to ensure aerated conditions. The effect of nutritional stress was determined in unaerated and aerated cultures after addition of nitrate (as potassium nitrate, 10 mmol/L), acetate (as sodium acetate, 10 mmol/L), citrate (as trisodium citrate, 1 mmol/L) and fructose (50 mmol/L) into the N_2 medium. To study the effect of nutrient deficiency, phosphate-free nitrate medium and N_2 medium diluted 2, 4 and

8 times with sterilized distilled water with nitrate (10 mmol/L) was used to estimate growth, akinete counts and amount of reserve products.

Growth and akinete frequency. Growth experiments were done at initial absorbance of 0.05; at regular intervals of 2 d the absorbance of the suspension was measured at 660 nm in a spectrophotometer and specific growth constant (k) was calculated according to Kratz and Myers (1955). The cultures were observed regularly under light microscope and the number of vegetative cells and akinetes was counted: akinetes as percentage of at least 1000 total cells at a time from a culture where mature akinetes were differentiated; the number of vegetative cells was determined using a hemocytometer, an average of 20 readings was taken.

Reserve products. Glg and CGP were estimated according to Sarma and Khattar (1986*a*). The amounts of Glg and CGP are expressed in fg per cell.

RESULTS AND DISCUSSION

The addition of nutrients (nitrate, acetate, citrate, fructose) to the N_2 medium under unaerated conditions enhanced the growth rate of the organism (reflected by the increase in k); in nitrate medium and nitrate medium without phosphate k increased to 0.37 and 0.40, respectively, from 0.19 (control medium; Table I). The k value after growth in 1:8 diluted medium was 0.19, similar to the k in the N_2 medium. In all nutrients the period of akinete formation was shortened; simultaneously, a relative amount of akinetes increased.

Table I. Growth (specific growth constant, k), initiation period (d), period of differentiation (d) and frequency (%) of akinetes in unaerated (*first lines*) and aerated (*second lines*) cultures of *A. torulosa*

Medium	k	Initiation, d	Free akinetes	
			time, d	frequency, %
Nitrate-free	0.19	8	20	15 ± 1.0
	0.21	–	–	0
Nitrate	0.37	4	12	21 ± 1.0
	0.70	–	–	0
Acetate	0.31	4	12	39 ± 4.0
	0.47	–	–	0
Citrate	0.32	4	12	28 ± 4.0
	0.49	–	–	0
Fructose	0.23	4	12	13 ± 0.9
	0.40	–	–	0
Nitrate–phosphate	0.40	4	12	45 ± 5.0
	0.40	–	–	0
N_2 diluted 1:2	0.15	4	12	20 ± 1.0
+ nitrate	–	–	–	–
1:4	0.22	4	12	25 ± 1.4
	–	–	–	–
1:8	0.19	4	14	85 ± 6.0
	–	–	–	–

The stress caused by deficiency of nutrients also increased the frequency of akinetes (reflected by their higher percentage in the phosphate-deficient nitrate medium). This was also confirmed by the increased frequency of akinetes with progressive dilution of the medium (in 1:8 diluted medium 85 % akinetes were found).

Aerated cultures exhibited faster growth as shown by the increase of k to a maximum of 0.70 in nitrate-grown cultures, followed by citrate- (0.49) and acetate-grown (0.47) cultures. The k in nitrate enriched cultures without phosphate and fructose was found to be the same. Akinete differentiation, however, did not occur in aerated cultures under either conditions, nutrient-sufficient and -deficient.

While akinete differentiation in *A. torulosa* we observed during the late exponential phase, in *A. cylindrica* (Fay 1969; Simon 1977; Nichols *et al.* 1980), *Aphanizomenon flos-aquae* (Rother and Fay 1979) and *Nostoc* strain PCC 7524 (Sutherland *et al.* 1979) it was reported when growth of the organism ceased.

Phosphate depletion from the nitrate medium did not alter either *k* or the period of akinete differentiation. Phosphate deficiency, however, enhanced the number of akinetes which is in agreement with findings of Sarma and Khattar (1992) with the same organism who found that the absence of phosphate decreased the ability of the organism to fix nitrogen while the nitrate-utilizing system appeared to function normally.

In unaerated cultures the stress caused by either high concentrations (of nitrate, acetate, citrate and fructose) or dilution of the medium, and depletion of phosphate can be considered to lead to akinete differentiation. Aeration, on the other hand, relieves the organism from nutritional stress, as akinetes were not formed under aerated conditions.

Glg content under unaerated conditions was found to be maximum in nitrate cultures deficient in phosphate, followed by 4 and 8 times-diluted nitrate cultures and nitrate-supplemented cultures, respectively, at the vegetative growth stage. At the akinete initiation phase (day 4 under unaerated conditions), the Glg content was enhanced considerably from vegetative-cell phase on fructose. In cultures containing free akinetes the Glg content either increased or decreased depending on the medium components. Under aerated conditions, when the cells divide more rapidly, Glg content was found to be nearly one-half or less on the day corresponding to initiation when compared with unaerated cultures. With rapid growth the Glg accumulation decreased in the aerated cultures up to 12 d (Table II). Accumulation of Glg during akinete differentiation in *A. torulosa* shows that excess of supplemented carbon can be assimilated and stored in the cells. Moreover, Pelroy and Bassham (1972) demonstrated that $^{14}CO_2$ was rapidly converted into 'polyglucose' metabolites during a light–dark transition experiments with 4 unicellular bacteria.

Table II. Contents (fg per cell)[a] of glycogen (*first lines*) and CGP (*second lines*) in unaerated (akinete forming) and aerated (non-akinete forming) cultures of *A. torulosa* (2, 4, 12 d)

Medium	Unaerated			Aerated		
	2	4	12	2	4	12
Nitrate-free[b]	1.25	4.0	1.0	0.43	0.46	0.34
	5.6	10.2	3.0	2.5	2.9	1.80
Nitrate	1.46	0.74	0.80	1.79	0.27	0.49
	2.91	3.5	5.9	1.62	1.39	1.88
Acetate	0.85	3.5	1.39	0.64	0.34	0.83
	3.7	4.3	2.5	2.3	2.8	3.1
Citrate	1.14	1.59	1.31	0.76	0.37	0.38
	4.3	3.4	1.78	3.2	2.1	4.3
Fructose	1.36	4.3	3.5	1.73	0.95	0.25
	2.9	7.8	14.0	3.4	0.71	3.5
Nitrate–phosphate	4.1	1.16	1.53	3.4	0.38	0.58
	4.6	4.2	9.0	1.01	1.67	4.5
1 : 2 diluted + nitrate	0.25	1.0	0.5	–	–	–
	2.5	4.8	6.3			
1 : 4 diluted + nitrate	2.5	1.31	0.25	–	–	–
	4.0	3.3	8.3			
1 : 8 diluted + nitrate	2.5	0.54	0.36	–	–	–
	8.3	4.7	6.0			

[a]The variation in values of the two reserve products was <5 %.

[b]Cultures were subjected to the estimation of the reserve products after 4, 8 and 20 d; in other experiments, cells in vegetative (2 d), initiation (4 d) and mature akinete phase (12 d) were tested.

The accumulation of CGP, however, exhibited a different pattern. Under unaerated conditions, in vegetative cells, the CGP content was maximum in 1 : 8 diluted nitrate-supplemented cultures, followed by nitrate medium without phosphate, 1 : 4 diluted nitrate medium, citrate- and acetate-supplemented media. A slow and consistent increase was observed in the content of CGP from the vegetative cell phase (2 d) to the akinete phase (12 d). While nitrate-free cultures showed a maximum of 10.2 fg per cell on day 4, in the presence of nitrate the content of CGP increased to 5.9 fg per cell on day 12. In the case of acetate and

citrate supplemented cultures the content of CGP slightly decreased from its initial level of about 4 fg per cell (2 d) to 2.5 and 1.78 fg per cell (12 d), respectively. While in cells grown in nitrate medium deficient in phosphate where the CGP content nearly doubled with the differentiation of akinetes on 12 d, concomitant with the transformation of nearly 50 % cells into akinetes. Similarly, cells in 1 : 2, 1 : 4 and 1 : 8 diluted nitrate medium differentiated into akinetes and showed a gradual increase in CGP. The CGP increase to maximum levels in the vegetative cells appears to be prerequisite for cell differentiation into akinetes. The CGP content after 12 d coinciding with akinete phase was found to be maximum in fructose-grown cells, followed by nitrate medium deficient in phosphate and 1 : 4 diluted nitrate grown cells. In contrast, the CGP content of cells grown in all nutrients under aerated conditions was generally lower than in corresponding unaerated cultures.

CGP is regarded as an excellent molecular store of nitrogen (Carr 1988). We found a higher CGP accumulation during the initiation of akinete formation but the same was not observed in aerated cultures supplemented with all nutrients. It appears that the effect of both high nutrient concentrations or their decreased levels in diluted cultures is eliminated in aerated cultures, leading to a lower content of the two reserve metabolites in the absence of akinete differentiation. Thus, a more rapid growth was observed in aerated cultures supplied with all nutrients, the cells being in a continuous active phase of division (higher *k* values were found). Phosphate-deficient nitrate cultures and 8-times-diluted nitrate cultures which support a high frequency of akinetes also exhibited a higher CGP content. Similar results, though not associated with akinete formation, were reported after phosphate limitation in the unicellular forms of *Aphanocapsa* (Allen *et al*. 1980) and *Agmenellum quadruplicatum* (Stevens *et al.* 1981, who considered higher CGP accumulation in phosphate-deficient cultures to be attributed to the transformation of internal nitrogen reserves into CGP).

The synthesis of CGP is catalyzed by Arg-poly(Asp)-synthetase ('cyanophycin synthetase') involving the synthesis of a polyaspartate primer onto which arginine is inserted (Simon 1976). The mobilization of CGP in two species of *Anabaena* sp. showed that the major initial product of cyanophycin breakdown is aspartate–arginine dipeptide mediated by the enzyme 'cyanophycinase' (Gupta and Carr 1981). The variation in the levels of CGP and the formation–degradation balance reflects that the synthesis and utilization of CGP proceeds once the organism undergoes akinete formation. Thus of the two reserve metabolites (Glg and CGP) the latter seems to accumulate in larger amounts preceding akinete differentiation. These data suggest that nitrogen reserves (*e.g.*, CGP) play a crucial role enhancing the cell nitrogen content which results in a lower C : N ratio and leads to akinete differentiation. Our observations further support similar results of Sarma and Khattar (1993).

The accumulation of reserve products in higher amounts in unaerated cultures is associated with early cessation of growth and lower growth rate, leading to akinete differentiation; in unaerated cultures akinete differentiation seems to be a consequence of a nutritional stress. Aerated cultures, on the other hand, are characterized by higher growth rate, lack of akinete differentiation and lower amounts of both reserve products.

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