EFFECTS OF LOW TEMPERATURE ON TWO CYANOBACTERIA

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The effects of low-temperature (0°C) incubation on nitrogenase-catalyzed acetylene reduction, alkaline phosphatase activity and cellular ATP pool(s) have been studied in *Nostoc linckia* and *Nostoc muscorum*. The cultures incubated at 0°C showed higher enzymatic activities than those incubated at 25°C but the ATP content was lower at 0°C. Our experiments with chloramphenicol and rifampicin suggest that de novo protein synthesis is required for nitrogenase, but not for alkaline phosphatase even at low temperature. The increase in alkaline phosphatase activity at 0°C may possibly be due to enhanced catalytic activity of this enzyme. The ability to protect enzyme activities at 0°C implies that *N. linckia* and *Nostoc muscorum* may be tolerant to low-temperature cellular damage.

The effect of temperature on metabolic processes is well established (11) and follows a general pattern of increasing metabolic rate with increasing temperatures. The susceptibility to low temperatures varies greatly from organism to organism and depends on growth temperature. Some plants are highly susceptible to chilling temperature (18) while some bacteria are markedly sensitive; some of them even die when exposed to low temperature near 0°C (19, 22).

Cyanobacteria are procaryotic organisms, but also share the property of oxygenic photosynthesis characteristically found in eukaryotic algae and higher plants. They seem to represent an evolutionary link between bacteria and plants. The study on the effect of low temperature (near 0°C) on cyanobacteria could therefore increase our understanding of the mechanism of the chilling susceptibility of higher plants and cold shock of bacteria. Cyanobacteria are frequently exposed to alternating freeze-thaw cycles during the early spring and late fall seasons in temperate regions. However, the question of how cyanobacteria respond to freezing has received little attention. Ono and Murata (21) investigated the effects of chilling on photosynthetic activities of *Anacystis nidulans*. When the algal cells

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are exposed to low temperatures, the viability of cells declines (23), the photosynthetic O_2 -evolution, the electron transport and the phosphorylation diminish (7, 12, 23) and the light absorption spectrum of carotenoid changes (3). Glutamate and pteridines are released from the cells (7, 12) and the cytoplasmic membrane and the thylakoid membranes are morphologically altered (4) at low temperatures.

The effects of freezing have been studied only with nitrogenase extract preparations (5, 8, 28) and in vivo only with terrestrial cyanobacteria (6). There have been no reports describing the effects of low temperature (near 0°C) on in vivo nitrogenase and alkaline phosphatase activities in mesophilic cyanobacteria. The present study deals with the effect of low-temperature (0°C) incubation on in vivo nitrogenase and alkaline phosphatase activities and cellular ATP content of *Nostoc linckia* and *Nostoc muscorum*.

MATERIALS AND METHODS

Nostoc linckia and *N. muscorum* were grown axenically in an inorganic medium free from combined nitrogen (1), at a temperature of $25 \pm 1^\circ\text{C}$ in aerated ($15\text{--}20\text{ ml min}^{-1}$) culture tubes. Culture bath was illuminated with four F36T12/D/HO fluorescent lamps on either side of the bath at an average intensity of $300\ \mu\text{E m}^{-2}\text{s}^{-1}$. The growth was measured turbidimetrically in a Bausch and Lomb Spectronic-20 colorimeter at 660 nm. Dry weight and chlorophyll *a* were determined as described earlier (13).

Nitrogenase activity was measured by the acetylene-ethylene assay utilizing the nitrogenase-catalyzed conversion of acetylene to ethylene (27). The suspensions of 2 ml of whole cell cultures were placed into 8 ml rubber-stoppered vacutainer tubes containing 90% air + 10% C_2H_2 and placed in the shaker bath having facility for low-temperature (0°C) incubation. Ethylene was measured by injection of 0.5 ml of the gas-phase into an CIC-gas chromatograph fitted with R-Porapak column. N_2 at 40 ml min^{-1} was used as the carrier gas.

Alkaline phosphatase activity was estimated by the method of Ihlenfeldt and Gibson (10). Culture (4 ml) was centrifuged and treated with toluene (2% v/v). To this, 4.0 ml of Tris-HCl buffer (0.2 M; pH 8.2) was added and equilibrated for 10 min at 37°C in waterbath. The reaction was started by the addition of 0.2 ml of *p*-nitrophenyl phosphate (100 mM) and incubated for 30 min at 37°C . The reaction was terminated by the addition of 0.2 ml of 1 M K_2HPO_4 . The liberated yellow-colored *p*-nitrophenol was measured at 420 nm. Specific activity was expressed as $\text{nmol } p\text{-nitrophenol min}^{-1}\text{ mg protein}^{-1}$. Cellular ATP content was extracted using 4% trichloroacetic acid (TCA) and 2 mM EDTA by the method of Larsson and Olsson (15). ATP content was estimated by luciferine-luciferase assay method using LKB-1250 luminometer.

RESULTS AND DISCUSSION

Actively nitrogen-fixing cultures of *Nostoc linckia* and *N. muscorum* showed about 5 to 6% of heterocysts frequency and generation time of 25 h and 22 h, respectively. The cellular nitrogenase activities found in *N. linckia* and *N. muscorum* were 65 and 124 nmol C₂H₄ mg dry wt⁻¹ h⁻¹, respectively. However, when these cultures were incubated in the dark, a significant decrease in the activity was observed (Figs. 1, 2).

N. linckia and *N. muscorum* incubated at 0°C under light showed 60 and 68 nmol C₂H₄ formed mg dry wt⁻¹ h⁻¹, respectively. This activity was almost comparable to the control value at 25°C in *N. linckia*. However, *N. muscorum* showed about 45% less activity than the control value. When these cultures were incubated in dark at 0°C, *N. linckia* and *N. muscorum* showed an enhancement of about 95% and 45% of nitrogenase activity over control dark values at 25°C (Figs. 1, 2). Glucose (1%) stimulated nitrogenase activity in the dark-incubated cultures of both the strains. However, cultures incubated at 0°C when supplemented with

Fig. 1. Effect of low-temperature treatment on nitrogenase (acetylene reducing) activity of *Nostoc linckia*. Cultures were grown under nitrogen-fixing condition at 25°C in a light intensity of 300 $\mu\text{E m}^{-2} \text{s}^{-1}$. For this experiment, exponentially grown cultures were divided into three sets. First set was incubated under light (●—●); second was incubated under dark (▲); third set was incubated under dark spiked with 1% glucose (△). After 60 min, the light-incubated sample was divided into three sets and incubated under different indicated experimental conditions: Chloramphenicol (50 $\mu\text{g ml}^{-1}$) or rifampicin (25 $\mu\text{g ml}^{-1}$) at 25°C under dark or light (○); Dark, 0°C (●---●); Light, 0°C (x). Incubation and assay were performed at respective temperatures.

Fig. 2. Effect of low-temperature treatment on nitrogenase (acetylene reducing) activity of *Nostoc muscorum*. Experimental conditions are the same as Fig. 1.

Table 1. Effect of preillumination and chloramphenicol on acetylene reducing activity at 0°C of *Nostoc linckia* and *Nostoc muscorum*. Cultures were grown to exponential phase under 300 $\mu\text{E m}^{-2}\text{s}^{-1}$ light intensity at 25°C.

Condition	Acetylene reducing activity (nmol C ₂ H ₄ mg dry wt ⁻¹ 5 h ⁻¹)	
	<i>Nostoc linckia</i>	<i>Nostoc muscorum</i>
Light, 25°C	300	500
Dark, 25°C	70	140
0°C, light	250	200
*0°C, light	100	165
*0°C + glucose (1%), light	180	234
0°C, chloramphenicol (50 $\mu\text{g ml}^{-1}$), light	25	40

* Samples of 0°C and 0°C + glucose (1%) were darkened at 25°C for 5 h before acetylene addition. Other sets were incubated and assayed under indicated experimental conditions.

either chloramphenicol (50 $\mu\text{g ml}^{-1}$) or rifampicin (25 $\mu\text{g ml}^{-1}$) showed less acetylene reducing activity after an hour (Figs. 1, 2) and there was no increase in the activity even after 3 h of treatment. Addition of chloramphenicol and rifampicin alone indicates the involvement of de novo protein synthesis in the enhancement of nitrogenase activity at 0°C.

To test whether light has any effect on the nitrogenase activity of the cultures incubated at 0°C, samples were darkened for 5 h at 25°C and then incubated and assayed for acetylene reducing activity at 0°C (Table 1). Cultures of both the

strains incubated at either 0°C or at 25°C in presence of light exhibited higher nitrogenase activity in comparison to dark-incubated cultures. Furthermore, addition of glucose (1%) stimulated the nitrogenase activity of the darkened samples. Chloramphenicol supplementation inhibited the nitrogenase activity of both the strains.

Nitrogenase is generally inactivated at temperatures around 0°C. It appears that it may be the Fe-protein which exhibits greatest sensitivity to cold as well as to O₂. Dua and Burris (5) reported an inactivation of approximately 85% nitrogenase of crude extracts from *Clostridium* at low temperatures. However, cold lability varies from organism to organism as, even in pure state, the Fe-protein of *Klebsiella pneumoniae* nitrogenase is insensitive to the cold. Zumft and Mortenson (28) have suggested the possibility of structural changes in the ferredoxin protein component of bacterial nitrogenase. In the present study, high rates of nitrogenase activity were detected in low-temperature-incubated cultures. This implies that nitrogenase is capable of surviving at low temperatures. However, de novo synthesis of nitrogenase or other critical proteins is required at low-temperature incubations.

Prefreezing conditions (light or dark) and the glucose experiment indicate that photosynthetic products (carbohydrates) have a major impact on the nitrogenase activity during low temperatures. The light pretreatment would increase the potential for storage of carbon compounds whereas the dark pretreatment would promote the utilization of stored carbon compounds. Many reports have shown that freeze protection can be afforded by carbohydrates (17,25), organic acids (24), amino acids (9). The storage of organic compounds may raise cell's osmotic potential and hence play an important role in low-temperature protection. This study shows that glucose addition has a beneficial effect on nitrogenase activity of the low-temperature (0°C)-incubated cultures.

The ATP requirement for nitrogenase activity in cyanobacteria is normally supplied by photophosphorylation (26) and possibly by oxidative phosphorylation (16). Utilization of stored carbon compounds (under dark conditions) can supply energy for nitrogenase and other enzymes. Subsequently, we assayed total cellular

Table 2. ATP content of *Nostoc linckia* and *Nostoc muscorum* under different experimental conditions. ATP content was expressed as nmol ATP mg Chl *a*⁻¹. Cultures were grown under nitrogen-fixing conditions at 25°C and 300 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity. Incubations were performed for 5 h at respective temperatures (0 and 25°C) and then the ATP content was measured.

Condition	<i>Nostoc linckia</i>		<i>Nostoc muscorum</i>	
	0°C	25°C	0°C	25°C
Light	579	662	305	317
Dark	350	250	300	309
Dark + glucose (1%)	420	345	332	347
Dark + chloramphenicol (50 $\mu\text{g ml}^{-1}$)	345	217	253	267
Dark + rifampicin (25 $\mu\text{g ml}^{-1}$)	333	200	271	250

Table 3. Alkaline phosphatase activity of test organisms under different conditions. Cultures were grown under phosphate-starved conditions for 24 h and then incubated for 5 h at respective temperatures (0 and 25°C). Alkaline phosphatase activity was assayed at 37°C of all samples.

Condition	Alkaline phosphatase activity (nmol PNP ^a min ⁻¹ mg protein ⁻¹)			
	<i>Nostoc linckia</i>		<i>Nostoc muscorum</i>	
	0°C	25°C	0°C	25°C
Light	22.0	17.5	8.5	7.9
Dark	21.2	17.3	7.8	6.8
Glucose (50 mM)	15.5	10.5	8.3	5.8
Chloramphenicol (50 µg ml ⁻¹)	19.8	17.2	8.0	5.1
Rifampicin (25 µg ml ⁻¹)	21.8	17.7	7.0	6.0

^a PNP: *p*-Nitrophenol.

ATP content of both the cultures under different conditions (Table 2). Light-transferred cultures of both the test organisms showed higher ATP content at 25°C than those at 0°C. However, there was a marked difference (14%) in the ATP content of light-incubated cultures of *N. linckia* at both temperatures. Dark-incubated cultures had lower ATP content than light-incubated cultures of both the strains. Addition of glucose (1%) to dark-incubated cultures of either of the two strains showed a remarkable change in the ATP content with respect to temperature (Table 2). It is seen from Table 2 that *N. linckia* showed higher ATP content at 0°C and *N. muscorum* showed it at 25°C in dark-incubated cultures. ATP content of dark-incubated, glucose-supplemented cultures of *N. muscorum* was even higher than light-grown cultures. Chloramphenicol (50 µg ml⁻¹) and rifampicin (25 µg ml⁻¹) treatments had no effect on ATP content of both the strains. Although the studies by Bottomley and Stewart (2), Mullineaux et al. (20) and Kumar (14) suggested that ATP supply does not limit nitrogen fixation in vivo, it is not yet clear whether the supply of ATP or of reductant primarily controls the activity of dark nitrogen fixation in cyanobacteria. Our experiment indicates that it is not ATP content but rather the reductant which plays an important role at low-temperature incubation.

Table 3 shows the alkaline phosphatase activity of the test organisms incubated at 0°C and 25°C under various conditions. Light had no effect on alkaline phosphatase activity of any of the strains. However, low-temperature incubation (0°C) showed higher enzyme activity. Chloramphenicol and rifampicin treatment produced no effect, suggesting that no fresh protein for alkaline phosphatase is synthesized during the low-temperature treatment. The increase in alkaline phosphatase activity during low-temperature incubation may possible be due to enhanced catalytic activity of enzyme.

The ability of the test organisms to protect both nitrogenase and alkaline phosphatase enzymes at 0°C suggests that *N. linckia* and *N. muscorum* may be

tolerant to low-temperature cellular damage.

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