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Nitrogen fixation in endolithic cyanobacterial communities of the McMurdo Dry Valley, Antarctica

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ABSTRACT: Nitrogen is scarce in the sandstones of McMurdo Dry Valley, Antarctica, and the possibility for the input from precipitation is minimal. In endolithic communities dominated by phototrophs, nitrogen availability by nitrogen fixation may therefore be very important. To investigate this, nitrogenase activity of the whole communities (rock plus microorganisms) of Linneus Terrace, McMurdo Dry Valley, was measured by acetylene reduction assay and environmental factors affecting the enzyme activity were studied. The activity obtained from these studies presumably came from the dominant phototroph identified, the ancient unicellular cyanobacterium *Chroococcidiopsis*. We describe a comparative study of nitrogen fixation where one set of experiments was conducted under conditions likely to be present in the Antarctic from where the samples were obtained i.e., 275 lux and 5 °C, and the other under laboratory conditions with 2500 lux and 20 °C. Our studies revealed that there was a distinct diurnal pattern of nitrogenase activity in these endoliths. Nitrogen fixation thus seems to be a very important activity in the Antarctic endoliths where there is a permanently immobilized layer of phototrophic cells exposed to extreme environmental conditions. It is probable that, although there is a drastic reduction in the photosynthesis active radiation of about 1-3% photons reaching the phototrophic region of the endolith for photosynthesis during the day, it is this that provides the energy for nitrogen fixation during the night. Also a heterotrophic mechanism involved in the nitrogen fixation process cannot be ruled out, as osmolytes are abundant in these environments to protect the cells from desiccation.

KEYWORDS: *Chroococcidiopsis*, nitrogenase activity, aerobic and anaerobic conditions

INTRODUCTION

The Ross desert of Antarctic can be characterized as hostile to life. With the exception of a few protected niches, such as cracks and depressions in rocks, the surface is practically abiotic. Two important communities of indigenous life forms are found which include the cyanobacteria and, less frequently, fungi and algae forming lichen associations that are in fact hidden[1–5]. The cryptoendolithic communities exist under the surface of rocks in the high mountains, in the valley, and as algal mats under the ice cover of permanently frozen lakes[6–10]. The dominant cyanobacterium in the cryptoendolithic communities of rocks is the unicellular cyanobacterium *Chroococcidiopsis* that is the most primitive organism of this group found where all other organisms fail to grow[7,8,11]. Water is the primary limiting factor, the source of which is melting snow. The microbes within the rocks survive because they are able to interact within their niche and either exploit their attributes or modify the niche to make it more suitable[5,8,9,12,13].

Most studies on nutrient uptake and cycling in endolithic communities of Antarctic cold deserts have dealt with carbon[14,15]. Reports of P metabolism have been discussed recently when Banerjee et al[8,9] demonstrated low light and low temperature adaptations for the phosphatase enzyme and high rates of P uptake by *Chroococcidiopsis* studied as whole communities. There is, however, a lack of knowledge regarding the N metabolism of these organisms. Having a clue now to the role of phosphatase enzyme activity being significant for providing P to the endolithic communities, it is interesting to study aspects of N_2 metabolism of the organisms inside the rocks. As snow water is unlikely to be the source of nitrates and other nitrogenous compounds and the lack of any animal life to provide organic sources of N[16], one speculation as to the source of N for these communities is N_2 fixation by the unicellular *Chroococcidiopsis*. Although it was believed that non-heterocystous and unicellular forms are unable to fix N_2, large number of reports prove otherwise. The first report on aerobic nitrogen fixation in *Gloeothece* followed by *Aphanothece* (now *Cyanothece* sp.) was in 1969 by Wyatt and Silvey[17], and followed by

work of Singh[18], Ni et al[19], Gallon and Stal[20], and Fay[21]. Other studies relate to interactions between nitrogen fixation and O_2^- and other diazotrophs[21,22] and the more specialized reviews of Huang and Grobbelaar[23] on *Synechococcus* RF-1, *Trichodesmium*[24], and cyanobacterial mats[25–29] all point to the fact that non-heterocystous filamentous and unicellular cyanobacteria are capable of N_2 fixation.

It is pertinent to point out that microbes living inside Antarctic rocks share several features with the immobilized cells in those laboratory systems where limited metabolic activity is required. Inspired by such considerations, an attempt was therefore made to study nitrogen fixation by the unicellular *Chroococcidiopsis* using whole communities in rocks of the Antarctic.

MATERIAL AND METHODS

Rock samples

The Antarctic rock samples referred to as whole communities in the present manuscript came from Beacon sandstone boulder in the Linneus Terrace, McMurdo Dry Valley on the NNE flank of the Apocalypse peak. The dominant phototroph in the rocks was the unicellular cyanobacterium *Chroococcidiopsis* although a few coccoid bacteria were also present. The pH of the rock was 9.2. Nitrate, nitrogen, and total phosphorus were not detected. The total nitrogen was 0.001 µg/g and chlorophyll was present at 7.56 µg/g within the rock.

Preparation for laboratory assay

Because of the limited amount of rock material it was decided simply to use the mass of rock to standardize the amount of material in an assay rather than some measure of biomass such as lipid phosphate adopted by some authors. In this study, however, measurement of chlorophyll a content was made for the samples (1 g rock) with *Chroococcidiopsis*, and this value was used to calculate the nitrogenase activity (mol C_2H_4/µg Chl a/g rock/h).

Rock samples containing *Chroococcidiopsis* communities were homogenized lightly and the mass of the rock plus community used in the acetylene reduction assay was used to standardize the amount of community present. Rocks were fragmented in a shaded part of a cold culture room ($\sim$10 µmol photon $m^{-2}\,s^{-1}$) with a geological hammer and washed chisel until the material appeared relatively uniform. The process was carried out using a washed high-sided tray to prevent loss and the tray was housed in ice. Gloves were worn to prevent contamination. Homogenized material of approximately 1 g was placed in vacutainer tubes for acetylene reduction assay (ARA) and chlorophyll a estimation. The exact mass for each assay was determined at the end of an experiment. These precautions were taken to maintain the low temperature conditions likely to be present in the Antarctic so that the nitrogenase and other proteins were not exposed to increase in temperatures that could alter the enzyme activity.

Experimental design

Experiments were mainly divided into 2 parts, one set in which the vacutainer tubes containing the samples were kept under aerobic conditions and the other in which the vacutainer tubes containing the samples were kept in evacuated tubes filled with 99% N_2 and 1% CO_2 to produce anaerobic conditions. These 2 sets of tubes were then separated in light and dark conditions and different temperatures (5 and 20 °C). Due to the paucity of rock material it was possible to carry out only 3 replicates for each experiment and each experiment was repeated 3 times to check for artifacts.

Chlorophyll a analysis

Chlorophyll a was extracted in 80% acetone from each sample tube after the ARA estimation was done for each hour of the corresponding nitrogenase activity. This gave the exact value of Chl content for estimation of nitrogenase activity. Chlorophyll a concentration was calculated by measuring the absorbance at 663 nm[30].

Nitrogenase activity

Nitrogenase activity was measured by ARA[31]. Whole community rock samples (1 g) were taken in 7 ml vacutainer tubes (Becton, Dickinson, and Rutherford). For anaerobic conditions the tubes were thoroughly evacuated, and 99% N_2 + 1% CO_2 was injected by a hypodermic syringe. Acetylene concentration was kept at 10% for both aerobic and anaerobic analysis. The light intensity and temperature have been specifically mentioned in the tables. Unless otherwise stated light conditions in this communication throughout the results refer to low fluence rates of 275 lux, a light intensity similar to what can be expected inside the rocks of the cryptoendoliths of the Antarctic. For maintaining the low temperature and low light conditions, a controlled Remi orbital shaking incubator was used. The ethylene formed was determined in a Sigma 300 Gas liquid chromatograph fitted with a Poropak R column and flame ionization detector. Dark treatment was given by wrapping the vials twice in aluminium

Table 1 Nitrogenase activity (mol C_2H_4/µg Chl a/g rock/h) of whole community under aerobic conditions.

h	20 °C		5 °C	
	Light	Dark	Light	Dark
2	-	-	-	1.05 ± 0.002
4	-	0.095 ± 0.001	-	1.66 ± 0.001
8	-	1.15 ± 0.002	-	1.91 ± 0.002
12	-	1.165 ± 0.03	-	1.97 ± 0.02
24	0.097 ± 0.002	1.20 ± 0.004	0.099 ± 0.003	2.10 ± 0.015

Results in this table and the next are mean $\pm$ SD of 3 individual experiments with $n = 3$ for each experiment.

Table 2 Nitrogenase activity (mol C_2H_4/µg Chl a/g rock/h) of whole community under anaerobic (99% N_2 + 1% O_2) conditions.

h	20 °C		5 °C	
	Light	Dark	Light	Dark
2	-	1.07 ± 0.001	-	1.5 ± 0.003
4	1.62 ± 0.005	1.69 ± 0.005	1.85 ± 0.004	2.69 ± 0.005
8	1.95 ± 0.008	2.03 ± 0.02	2.15 ± 0.01	2.85 ± 0.03
12	1.98 ± 0.007	2.08 ± 0.04	2.25 ± 0.07	2.89 ± 0.06
24	2.09 ± 0.006	2.24 ± 0.04	2.40 ± 0.005	2.95 ± 0.04

foil. All the gases of high purity were obtained from Indian Oxygen Limited, Bombay.

The physiochemical parameters of the rocks were determined by standard methods[32]. Statistical analysis consisted of mean values and standard deviation that are given in the tables. Means were compared using standard t-tests at $\alpha = 0.05$.

RESULTS

Table 1 shows the nitrogenase activity measured as acetylene reduction of whole communities of rocks under aerobic conditions. After 12 h in the light, or 2 h in the dark, there was no nitrogenase activity detected at 20 °C. Nitrogenase activity was detected in the dark after 4 h and kept on increasing until 24 h. Under aerobic light conditions, activity was reported after 24 h. Nitrogenase activity in the dark was higher than what was observed in light. When a similar experiment was conducted at 5 °C and 275 lux light intensity, the nitrogenase activity was higher than that at 20 °C. C_2H_4 reduction was detected after 2 h in dark, but after 4 h at 20 °C. In light, however, the activity was detected at 24 h and was less than that of the dark conditions. The activity at 5 °C in the dark was about 75% higher than that at 20 °C after 24 h ($P < 0.05$). At 275 lux the C_2H_4 reduction in activity was detected at 24 h and the activity at 5 °C was about 17% higher than at 20 °C ($P < 0.01$).

Table 2 shows the nitrogenase activity of rock samples under anaerobic conditions. A striking observation was that the C_2H_4 reduction was significantly enhanced under anaerobic conditions in both light and dark compared to aerobic conditions at these temperatures. After 24 h in light, there was nearly a 46% increase in nitrogenase activity under anaerobic conditions compared to aerobic conditions ($P < 0.05$) at 20 °C. Similarly in the dark under anaerobic conditions at 20 °C after 24 h there was a 54% increase compared to aerobic conditions ($P < 0.01$). A general observation was that nitrogen fixation was detected earlier and had higher values in the dark compared to in the light at all temperatures studied (5 °C and 20 °C). Nitrogenase activity was always higher under anaerobic conditions compared to aerobic conditions.

The ARAs were also conducted at 5 °C under the anaerobic conditions. In both light and dark the nitrogenase activity was much higher than that observed at 20 °C. This increase was 15% in light and 33% in dark at 24 h at 5 °C ($P > 0.01$, $P > 0.05$). For nitrogen fixation at 5 °C, a 47.1% increase was observed under anaerobic conditions compared to aerobic conditions at 24 h in light ($P > 0.05$). Similarly, in the dark at 24 h, a 42% increase was observed under anaerobic conditions at 5 °C ($P > 0.01$).

Table 2 also shows the nitrogenase activity at 20 °C and 5 °C at the light intensity of 2500 lux (the light intensity of the culture room) in the laboratory conditions. Under aerobic conditions no enzyme activity was detected even at 24 h. Very negligible activity was observed under dark anaerobic conditions at 24 h but the activity was higher at 5 °C compared to 20 °C.

DISCUSSION

The observations of this present study offer some pertinent and possible answers to the phenomenon by which the cryptoendolithic cyanobacteria within the Antarctic rocks obtain their nitrogen requirement. Analysis of total nitrogen and nitrate reveals that there is negligible total nitrogen and no nitrate present in the rocks. Therefore it is evident that some other processes must be operating to provide nitrogen for the phototrophs present. Although studies on hot semi-arid lands and deserts by some authors suggest that nitrogen might not be the limiting factors in those habitats, our results prove otherwise[6,14].

The cyanobacterial strain *Chroococcidiopsis* used in this study is present in the phototrophic zone of the Antarctic endolith. It is a non-heterocystous unicellular strain. A major factor that has attracted scientists to the study of nitrogen fixation in non-heterocystous cyanobacteria has been the apparent paradox of these organisms that simultaneously fix nitrogen and pho-

toevolve O_2. In the present study, distinctly higher activity in the dark and under anaerobic conditions suggests that the spatial separation of N_2 fixation and photosynthesis may be very important for naturally occurring *Chroococcidiopsis* under endolithic conditions. Anaerobic or microaerobic conditions may be prevailing at the interphase of the photoautotrophic zone, where this cyanobacterium appears to be permanently immobilized, and the sandstone layer above it. The phototrophic zone is few millimetres below the surface of the rocks, and therefore the O_2 concentration available to the cyanobacterium may be less than at the surface, and the actively respiring bacteria may remove some of it, thus creating a microanaerobic condition preventing the oxygen sensitive nitrogenase from being inactivated. There is probably a gradation of tolerance to oxygen among non-heterocystous N_2 cyanobacteria and some strains are more sensitive to O_2 than others[33].

Mechanisms the *Chroococcidiopsis* inside the rocks could use to minimize the deleterious effects of O_2 on N_2 fixation may include behavioural strategies, physical barriers, and metabolic strategies. Behavioural strategies include avoidance of O_2, a strategy that is practiced by those cyanobacteria fixing N_2 under anoxic or microxic conditions such as the cryptoendolithic niche where the prevailing concentrations of O_2 is low.

A number of metabolic strategies may be employed by non-heterocystous cyanobacteria and *Chroococcidiopsis* in particular in order to cope with O_2. For example, metabolic consumption of O_2 seems of great importance in maintaining a relatively low concentration of intracellular O_2. In many cases, the pattern of respiration parallels that of N_2 fixation in cultures grown under alternating light and darkness or under continuous illumination. O_2 consumption is the sum of that of all individual oxygen consuming reactions. These include substrate level oxidase activity or oxygen consumption linked to aerobic electron transport chains. Such chains may have a large number of different terminal oxidases, and a variety of electron donors. Both carbon linked and H_2 linked respiratory oxygen consumption are known among non-heterocystous cyanobacteria[22].

Perhaps the most intriguing possibility to have emerged in recent years is that nitrogenase may participate in protecting itself from inactivation by oxygen. Nitrogenase is a versatile enzyme and can reduce a number of substrates like C_2H_4. In vitro, when nitrogenase is present at a concentration greater than four times that of O_2, the reduced form of Fe-protein of nitrogenase reduces O_2 to H_2O_2 and H_2O. In the second stage, reduction of H_2O_2 to H_2O is very slow and catalase and peroxides may be required to prevent accumulation of H_2O_2 in vivo. When the concentration of oxygen exceeds that of the Fe-protein, O_2 is reduced to O_2^- that inactivates nitrogenase. Thus whether the ability of nitrogenase to consume oxygen could protect itself from inactivation (autoprotection) depends critically upon the prevailing relative concentration of O_2 and Fe-protein. Since the microenvironment of endolithic *Chroococcidiopsis* is deep seated inside the rocks, there could be O_2 depletion to a certain extent which would promote autoprotection of nitrogenase and stimulate nitrogen fixation as in the observations in this work. Also, the energy supply for nitrogen fixation derives mainly from photosynthesis, but in the case of the whole communities in the present study, light is a very important limiting factor. Approximately only 1–3% of surface photosynthesis active radiation (PAR) reaches the phototrophic zone of the Antarctic endoliths, which are a few photons per day. Considering that in the phototrophic zone the growth of *Chroococcidiopsis* is quite luxuriant, this phenomenon could have two possible explanations: *Chroococcidiopsis* in the Antarctic endoliths possesses special accessory pigment for harvesting the available PAR, or the chloroplast or thylakoids of the cryptoendoliths are morphologically adapted to extreme conditions. Another explanation of higher nitrogenase activity in the dark compared to light in both aerobic and anaerobic conditions is that *Chroococcidiopsis* could be driving a heterotrophic N_2 fixation process using the osmolytes that are generally sugars and are associated with these organisms to protect the cells from desiccation and osmotic shocks in a chemoautotrophic pathway for reductant and energy supply to drive nitrogenase activity. No activity under high light fluencies points to the fact that *Chroococcidiopsis* is a low light species and cannot tolerate high light intensities and their photosynthetic pigments may become bleached.

Although further work is needed to assign a more accurate elucidation of the working of nitrogenase in cryptoendoliths, it is nevertheless evident that nitrogenase activity regulates nitrogen availability to the phototroph in general and *Chroococcidiopsis* in particular in the endolithic habitat of the Antarctic. This unique and interesting niche offers more questions than it has yielded answers to date and should prove to be a fertile research area for the future.

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