DIAZOTROPHIC GROWTH AND NITROGENASE ACTIVITY OF CYANOBACTERIA FROM THE PATOS LAGOON ESTUARY, SOUTHERN BRAZIL

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ABSTRACT

Several samples of cyanobacteria were collected from freshwater, brackish and marine environments of, and near, the Patos Lagoon estuary in Southern Brazil. Laboratory isolation and purification techniques were applied to obtain 25 axenic cultures in medium free of combined nitrogen. From these, 10 cultures were selected and their diazotrophic growth and nitrogenase activity were followed for 30 days of cultures in a mineral freshwater medium (VB-S) and in a saltwater medium (ASN-III). Diazotrophic growth and nitrogenase activity were monitored by the chlorophyll a content and acetylene reduction rates, respectively.

Cultures of Nostoc strain RSA, 8601, Calothrix strain RSA, 8601, Nostoc strain RSJ 8502, Microchaete strain RSJ 8501 and Anabaena strain RST 8701, all of which are heterocystous, isolated originally from fresh and brackish water, showed an increasing diazotrophic growth. These cultures also presented higher levels of nitrogenase activity at the lag phase of growth. The two non-heterocystous strains, Synechocystis RSSM 8801 and Oscillatoria RSSM 8801, originally isolated from marine waters, presented a diazotrophic growth comparable to the heterocystous cultures, although no nitrogenase activity was detected throughout the light and dark cycles.

Cultures of Anabaena strain RSC 8801, Mastigocladus strain RSC 8801, Lyngbya strain RSSM 8801 originally isolated from marine and brackish waters grew much more slowly and no nitrogenase activity was detected.

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These results suggest a division of the 10 cultures in the basis of the growth rate under the conditions tested. It is possible to conclude that such genera (particularly the heterocystous strains studied) from freshwater and brackish environments may be important as potential N\(_2\) fixers for laboratory studies regarding environmental conditions.

KEY WORDS: cyanobacteria, nitrogenase activity, diazotrophic growth.

1 - INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes, many species of which (including unicellular, filamentous non-heterocystous and filamentous heterocystous) are able to fix N\(_2\) (Rippka et al. 1979) and may have an important role in nitrogen cycling in coastal benthic environments (Capone, 1983). The present work aims to study cyanobacteria isolated from the Patos Lagoon (Brazil), an estuary characterized by a rich nutrient supply (Niencheski
and Windom, 1994) and distinct salt gradients (Kantin, 1983). Benthic cyanobacteria have been isolated from the margins of the Patos Lagoon and their ability to fix N₂ studied using the acetylene-reduction technique of Stewart et al. (1967). Although this technique has limitations, it is still a rapid, simple and useful method to assess nitrogenase activity. Investigations were performed in the field and in the laboratory under conditions approximating to those in the lagoon itself. Such an approach can provide useful information on the potential contribution of the cyanobacteria to the environment (Home and Commins, 1987).

The diazotrophic growth of Nostoc muscorum strain RSJ 8501 under conditions of combined nitrogen availability approximating to those in the lagoon (Yunes et al. 1990) has been previously reported. In the present work, this evaluation is extended to other benthic cyanobacterial isolates representing the variety of benthic habitats in the Patos Lagoon and its surrounding areas.

2 - MATERIALS AND METHODS

2.1 - isolation and culture conditions

The organisms investigated below were sampled from the estuarine margins of the Patos Lagoon and nearby areas, situated in the state of Rio Grande do Sul, Brazil (Fig. 1). Isolates were purified by successive plating on nitrogen-free growth medium. Samples from brackish and fresh waters were grown on liquid cultures of the VB-S mineral medium (Yunes & Melo, 1987) and salt water samples in the ASN-III⁰ mineral medium (Rippka et al., 1979). Strains were identified according to Desikachary (1959), classified following the nomenclature for cyanobacteria proposed by Rippka et al. (1979) and code-registered by the local and year of sampling. The axenic nature of the culture was controlled by microscopic examination. Isolates were grown in 1.8l batch culture at 25°C in either VB-S or ASN-III⁰ media, and 12h light/12h dark regime was imposed with a photon flux rate of 107 µE m⁻² s⁻¹ irradiance of the surface of the culture during the light period. Air was filtered through 25% H₂SO₄, 1% NaHCO₃ and distilled water, and bubbled into the cultures at a rate of 0.265l/min.
2.2 - Nitrogenase activity

The acetylene reduction assay (Stewart et al. 1967) was used to measure nitrogenase activity. Ten ml aliquots of growing cultures were sealed in 20ml vacumtainer tubes (NEW VACUO). Acetylene was injected to a 10% v/v of internal atmosphere and incubated for 1h at 25°C and under
107μE. m⁻¹ s⁻¹ of irradiation. Ethylene production was measured by injecting 1ml of the gas phase into a gas chromatograph (VARIAN MODEL 2485) with a flame ionization detector and a PORAPAK N COLUMN. All gases were super dried type (WHITE MARTINS). In situ nitrogenase activity tests were performed by using inverted funnels sealed with rubber stoppers and placed over cyanobacterial mats. Acetylene was injected through the rubber seals to a 1% v/v of the remaining atmosphere. After 3h of in situ incubation, 1ml of the gas phase was sampled into syringes which were then sealed and transferred to the laboratory. Ethylene production was measured as above.

2.3 - Diazotrophic growth

It was followed by the increase in chlorophyll a. Ten ml aliquots of culture were collected by centrifugation, resuspended in 5ml of methanol and incubated in the dark at 4°C for 24h. Absorption was recorded at 663mm and 750mm and the chlorophyll a calculated with the coefficient of 75 L.g. cm⁻¹ (Rieman, 1980).

3 - RESULTS AND DISCUSSION

3.1 - Diazotrophic growth

The diazotrophic growth was different in the ten strains examined (Fig. 2). The most rapid growth (as estimated by chlorophyll a concentration) was shown by cultures of Nostoc RSA₂8601 (Nostoc muscorum), Anabaena RST8701 (Anabaena verrucosa), Nostoc RSJ8502 (Nostoc punctiforme), Microchaete RSJ8501 (Microchaete tenera) and Calothrix RSA₂8601 (Calothrix brevissima). These organisms grew to a density greater than 1.2mg. chlorophyll a.ml⁻¹ after 30 days of culture period. Most notably, the two Rivulariacean genera, Microchaete RSJ8501 and Calothrix RSA₂8601 grew to a density of 3.0mg. chlorophyll a.ml culture⁻¹ during the same period. Although variable, these cultures tended to enter the log phase of growth after 15 days.

All the above are heterocystous strains isolated from fresh and brackish waters and their ability to grow diazotrophically is not surprising. A high efficiency of growth in a nitrogen-free mineral medium is a common feature of some Anabaena and Nostoc isolates either from paddy soils (Antarikanonda and Lorenzen, 1982; Chen, 1985) or from marine coastal environments (Gotto et al., 1979).
FIGURE 2 - Diazotrophic growth of the ten strains studied in nitrogen-free mineral media during 30 days. Growth was followed as µg chlorophyll-a per ml culture. Note the different scale for h, i and j.
Growth of the two marine non-heterocystous *Oscillatoria* RSSM 8801 and *Synechocystis* RSSM 8801 (*Aphanthece stagnina*) strains was slow in comparison to the heterocystous strains. Growth reached 1.0mg chlorophyll a:ml culture\(^{-1}\) after 30 days and both cultures have not left the lag phase of growth during the test. However, on these cyanobacteria (which lack heterocysts) the ability of *Oscillatoria* species to fix \(N_2\) under light and dark cycles is well documented (Khames et al., 1987) and are important as a nitrogen-fixing organism in oceanic surface waters (Carpenter, 1983). Similarly, *Synechocystis* species are widely distributed in coastal areas and some species can fix \(N_2\) (Rippka and Waterbury, 1977); therefore, the lower diazotrophic growth observed in these strains is possibly related to less favourable growth conditions, e.g. nutrient composition and physical factors. Recently, cultures of *Synechocystis* RSSM 8801 (*Aphanthece stagnina*) have been successfully grown on nitrate added medium (our own observation).

Cultures of *Anabaena* RSC 8801 exhibited an even lower growth than the cultures above. The strains *Lyngbya* RSSM 8801 and *Mastigocladus* RSC8801 (*M.laminosus*) have not shown any ability to grow diazotrophically under the conditions tested. The growth of the marine *Anabaena* RSC8801 compares poorly with that of the freshwater *Anabaena* strain (*Anabaena* RST8701). One reason for this may be the higher temperature required for growth in some marine *Anabaena* species (Gotto et al. 1979). Although the occurrence of *Lyngbya* in estuarine and coastal environments has been frequently reported (Baker, 1987; Bebout et al., 1987), there is little information on its ability to fix \(N_2\) (Stewart, 1968). *Mastigocladus* RSC8801 has been reported to fix \(N_2\) (Fogg, 1951); however, this ability is restricted to temperatures above 45\(^{\circ}\)C (Stevens et al., 1985) and the low temperatures used in the present study may explain the poor growth of *Mastigocladus* RSC8801. In the Patos Lagoon, the water temperatures rarely exceeds 30\(^{\circ}\)C and as such, the importance of diazotrophic growth of this *Mastigocladus* strain may be minor.

3.2 - Nitrogenase activity

Nitrogenase activity (expressed per ml of culture) was detectable in five heterocystous strains only (Fig. 3) and was higher in *Microchaete* RSJ8501, *Nostoc* RSA\(^{2}\)8601 and *Nostoc* RSJ8502. *Calothrix* RSA\(^{2}\)8601 and *Anabaena* RST8701, among them, exhibited lower levels of nitrogenase activity supported by the photoautotrophic growth regime. Photosynthesis in the vegetative cells provides sufficient ATP and reductant to sustain nitrogenase activity in heterocysts (Bottomley and Stewart, 1977). Heterocysts are specialized cyanobacterial cells which lack photosystem II and provide
ideal conditions for nitrogenase synthesis and activity (Haselkorn, 1978).

![Graphs showing nitrogenase activity](image)

**FIGURE 3** - Nitrogenase activity assayed as μmol of acetylene reduced per ml of culture (□) and as a ratio of chlorophyll-a concentration per ml of culture (■) during 30 days of growth.

However, this specialization is not provided in non-heterocystous species. Their nitrogenase and photosynthesis enzymes are restricted to the same vegetative cells and, in this case, their diazotrophic habillity is
maintained by several mechanisms (Gallon, 1981). In the other five cultures, nitrogenase activity could not be detected in either the light or the dark phases of the diurnal illumination regime. In the case of Oscillatoria RSSM8801 and Synechocystis RSSM8801 which had a lower diazotrophic growth (Fig. 2), the lack of detectable nitrogenase activity may be due to inhibition by sampling procedures on insufficient volume of cells. In the other cultures where diazotrophic growth was undetected, a limitation of N₂-fixation by sub-optimum temperatures (Mastigocladus RSC 8801 - Stevens et al., 1985) or oxygen inhibition (Lyngbya species - Gallon, 1980) may be responsible for the lack of nitrogenase activity.

3.3 - Nitrogenase activity and pigment concentration

In N₂-fixing cultures, the ratio of nitrogenase activity to chlorophyll a concentration decreased during the 30 days of culture, except for Microchaete RSJ8501 and Nostoc RSA₂8601 cultures (Fig. 3). In most cases, a higher ratio was found in the first 10 days of culture, followed by a sharp decrease which led to a steady and lower ratio towards the end of 30 days of culture. The increase observed in Microchaete RSJ8501 and Nostoc RSA₂8601 nitrogenase activity (as a chlorophyll a ratio) until the 20th day of culture is explained by the late start of log phase of growth already mentioned.

If this model of nitrogenase activity is compared to diazotrophic growth, it resembles an opposite feature of the two-phase growth model previously presented (Fig. 2). This may suggest that the higher rates of nitrogenase activity in the first days of cultures promoted sufficient nitrogen reserves to allow higher rates of cell division and pigment synthesis throughout log phase. This correlation has been previously demonstrated for chlorophyll a and also phycocyanin synthesis in both unicellular (Gallon et al., 1975) and heterocystous (Thomas, 1972) cyanobacteria. Such processes may also be relevant for growth in the environment.

3.4 - In situ nitrogenase activity

*In situ* nitrogenase activity was estimated in cyanobacterial mats of several sites on the Patos Lagoon margins. At most of these sites, a mixture of cyanobacteria species were present. In the brackish and freshwater environments of Justino bay and in the EMBRAPA rice fields, Nostoc, Anabaena and Calothrix species dominated. In marine habitats (salt marsh), Oscillatoria, Lyngbya and other unicellular and filamentous non-heterocystous species were found. Values for acetylene reduction at sites where nitrogenase activity was detected ranged from 0.0423 nmol C₂H₄ cm⁻² h⁻¹ to 1.3572 nmol C₂H₄ cm⁻² h⁻¹ (Tab. 1). At several sites where Oscillatoria and other non-heterocyst species

were dominant, nitrogenase values were lower than 0.01 nmol C₂H₄ cm⁻² h⁻¹.

**TABLE 1 - Nitrogenase activity assayed as nmol C₂H₄ cm⁻² h⁻¹ of acetylene reduced and the predominant genera of cyanobacteria found on the margins of two sites in the Patos Lagoon estuary. The data shown are the average of 5 determinations on each test.**

<table>
<thead>
<tr>
<th>SITE OF ASSAY</th>
<th>nmol C₂H₄ cm⁻² h⁻¹</th>
<th>PREDOMINANT GENERA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown mat on flooded margins, Justino bay.</td>
<td>0.245</td>
<td><em>Nostoc, Anabaena</em> and Oscillatoria.</td>
</tr>
<tr>
<td>Green mat on wooden surface under water, Justino bay.</td>
<td>0.230</td>
<td>Calothrix</td>
</tr>
<tr>
<td>Green mat on flooded margins, Justino bay.</td>
<td>1.357</td>
<td><em>Nostoc and Anabaena</em></td>
</tr>
<tr>
<td></td>
<td>0.070</td>
<td><em>Nostoc, Anabaena</em>, Oscillatoria and Lyngbya.</td>
</tr>
<tr>
<td>Green mat on the upper littoral margins, salt marsh, Cassino beach.</td>
<td>&lt; 0.010</td>
<td>Oscillatoria and Chlorogloea</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.010</td>
<td>Oscillatoria, Anabaena</td>
</tr>
<tr>
<td></td>
<td>0.303</td>
<td><em>Microcystis and Spirulina</em></td>
</tr>
<tr>
<td></td>
<td>&lt; 0.010</td>
<td><em>Nostoc and Oscillatoria</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oscillatoria, Microcystis, Anabaena and Spirulina</td>
</tr>
</tbody>
</table>

Likewise, previously reported field data on nitrogenase activity in cyanobacterial mats at coastal and estuarine sites (Gotto et al., 1981; Huber, 1986) and salt marshes (Stewart et al., 1978) have been within the same range. There are however a wide range of potential sources of errors in estimating nitrogenase activity in the field; notably temperature (Whiting and Morris, 1986), light and diel variations (Huber, 1986; Bebout et al., 1987), presence of nitrogen salts and oxygen (O₂). Furthermore, the acetylene reduction technique has limitations on accuracy (Dicker and Smith, 1980) and calibration and interpretation (Seitzinger and Gaber, 1987). As such, it would
be unwise to draw firm correlations between in situ and laboratory data. However, despite the limitations, nitrogenase activity was recorded in cyanobacterial mats where the studied strains were predominant and it suggests that these strains may play a role as diazotrophs in the estuarine area studied.

4 - CONCLUSION

Ten cyanobacterial strains isolated from the Patos Lagoon estuary can be grouped on the basis of growth rate under Nitrogen-free culture conditions. It is possible to conclude that such heterocystous genera from brackish and freshwater environments may be useful for laboratory studies and potentially important as nitrogen-fixing organisms, in their original habitats.

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REFERENCES


