Induction of sporulation by sulphate limitation in *Nostoc ANTH*, a symbiotic strain capable of colonizing roots of rice plants

Omarlin Kyndiah and Amar Nath Rai*

Department of Biochemistry, North Eastern Hill University, Shillong 793 022, India

Received 1 June 2005; revised 19 January 2006; accepted 29 March 2006

*Nostoc ANTH* is a symbiotically compatible cyanobacterium that associates with rice plants and carries out associative N$_2$-fixation. Investigations were carried out to induce profuse sporulation in the cyanobacterium for use as inocula in rice paddies. Impacts of pH and temperature changes, addition of various carbon sources and limitation of phosphate and sulphate on akinete formation were studied. Among these, only phosphate and sulphate limitation induced akinete formation in *Nostoc ANTH*. Under both the conditions all vegetative cells eventually became akinete. However, induction of akinete differentiation was quicker and resulted in more profuse akinetes differentiation in response to sulphate limitation than to phosphate limitation. These akinetes showed long-term viability (up to 5 years) and excellent germination frequency (90-95%). This is the first report on induction of akinete formation by sulphate limitation.

**Keywords:** *Nostoc ANTH*, cyanobacteria, akinetes, akinete differentiation, sulphate limitation

**IPC Code:** Int. Cl.8 C12N3/00

**Introduction**

Cyanobacteria are an ancient and diverse group of gram-negative eubacteria characterised by higher-plant type oxygenic photosynthesis	extsuperscript{1}. They occupy a wide range of habitats as free-living as well in symbiosis with other organisms	extsuperscript{2-6}. The potential role of diazotrophic cyanobacteria as biofertiliser in rice paddies has long been recognised	extsuperscript{7-8}. In recent years the use of diazotrophic cyanobacteria as biofertiliser is being popularised in several countries including India	extsuperscript{9-10}. A major concern in distributing cyanobacterial inocula to farmers is the high mortality of cyanobacterial cultures during storage and transport under field conditions. Akinetes are likely to serve as ideal inocula since they can withstand adverse conditions, can be stored and transported in dry form, and shall not require special storage conditions or packaging. It is, therefore, of great interest to devise strategies for triggering of akinete differentiation in selected diazotrophic cyanobacteria that may lead to quick and profuse formation of akinetes endowed with high viability and germination efficiency.

Environmental and nutritional factors which include limitation of nitrogen, carbon, iron, trace elements, and light have been reported as trigger for sporulation in cyanobacteria. In the past, several authors have reported that phosphate limitation is a major trigger for akinete formation in *Anabaena variabilis*, *A. cylindrica*, *A. circinalis* and *Nostoc linckia*	extsuperscript{11-17}. However, cyanobacterial strains maintained in laboratory tend to accumulate polyphosphate reserve (polyphosphate bodies) that takes long to deplete. This makes it difficult to create phosphate limitation and trigger akinete formation quickly.

In the present communication we report for the first time that sulphate-limitation triggers profuse akinete formation in *Nostoc ANTH*, a symbiotically compatible heterocystous N$_2$-fixing cyanobacterium known to associate with rice plants and carry out associative N$_2$-fixation	extsuperscript{18}. Such cyanobacterial strains have high potential as biofertiliser in rice cultivation and can be applied in the form of akinetes having high viability and efficient germination. In addition, this study demonstrates that sulphate limitation is a better trigger than phosphate limitation for akinete formation in *Nostoc ANTH*.

**Materials and Methods**

**Organism and Culture Conditions**

*Nostoc ANTH* was grown from axenic stock culture in N$_2$-medium (BG-11$_0$ medium	extsuperscript{19}) at 25° C with a light intensity (photons fluence rate) of 50 µmol photons.m$^{-2}$.s$^{-1}$. As and when required, the N$_2$-
medium was supplemented with 5 mM KNO₃ (nitrate-medium) or 2 mM NH₄Cl (ammonium-medium) as sources of combined nitrogen. The medium was always buffered with equimolar concentration of HEPES [4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid]. The pH of the medium was adjusted to 7.5 before autoclaving.

**Culture Condition for Akinete Differentiation**

Cultures of Nostoc ANTH were washed three times with BG-11₀ medium minus MgSO₄ and allowed to sporulate in the same medium lacking MgSO₄. The medium was supplemented with equimolar concentration of MgCl₂ to counter the effect of reducing the concentration of MgSO₄ so that the combined cation and anion concentration remained the same in all the cultures. As and when required, the medium was supplemented with 5 mM potassium nitrate or 2 mM ammonium chloride and buffered with equimolar concentration of HEPES. Sporulation experiments were also conducted at different temperature (20°, 25°, 30°, 35°, 45° or 50° C) and pH (5, 7.5, 9 or 11). The start of akinete differentiation was taken to be the time when akinetes first appeared. The end of akinete differentiation was taken to be when maximum number of cells became akinetes and no further akinete differentiation occurred.

**Storage Condition for Akinete**

Akinete population was centrifuged and the pellet containing akinetes washed. The pellet was air dried and stored at room temperature.

**Culture Condition for Akinete Germination**

Akinete population was washed twice and resuspended in fresh BG-11₀ medium at a concentration of 2 x 10⁶ akinetes mL⁻¹ and incubated in light at 50 μmol photons.m⁻².s⁻¹. As and when required, KNO₃ (5 mM) or NH₄Cl (2 mM) was added as sources of combined N.

**Akinete and Heterocyst Frequency and Akinete Viability**

Heterocyst and akinete frequency was calculated as percentage of total cell population by light microscopically. The percentage of germinate akinete was determined by examination of at least 1000 akinetes under light microscope. Akinetes that failed to produce germling and remained as single cell, were considered non-viable.

**Chlorophyll a and Protein Estimation**

Chlorophyll a was extracted in 90% methanol in darkness at 4°C. The absorbance at 663 nm was measured using a Beckman DU-530 Spectrophotometer and chlorophyll a concentration calculated according to Mackinney²⁰. Protein was measured according to Lowry et al.²¹

**Oxygen Exchange**

Oxygen evolution/consumption was measured polarographically using a Clark-type oxygen electrode installed in a 3 mL Plexiglass container with magnetic stirrer (Rank Brothers, England). Three mL cyanobacterial culture was added to the sample chamber of the non-polarised electrode and allowed to equilibrate for 5 min while stirring. The electrode was then polarised and the linear rate of oxygen evolution was obtained in light (100 W tungsten). The light intensity at the surface of the sample chamber was 50 μmol photons.m⁻².s⁻¹. Oxygen consumption was measured in dark with the chamber wrapped with aluminium foil. The rate of oxygen evolution and consumption is expressed as nmol O₂ evolved/consumed.min⁻¹.mg⁻¹ protein.

**Enzyme Activities**

Nitrogenase activity was measured as ethylene production using acetylene reduction assay.²² Glutamine synthetase (transferase) assay was essentially as described by Sampo et al.²³ except that CTAB (alkyltrimethylammoniumbromide) permeabilised cells were used. Nitrate reductase activity was measured in situ using CTAB-permeabilised cells.

**Microscopy**

Olympus microscope fitted with a JVC digital video camera was used. Microphotographs were taken using excitation filter (BP 545-580 nm). Heterocysts and akinetes did not fluoresce due to lack of phycobiliproteins while vegetative cells gave strong fluorescence. Akinetes were distinguishable from heterocysts since the latter contained distinct polar nodules (Fig.1).

**Results and Discussion**

Various factors that may trigger akinete formation in Nostoc ANTH were tested in the present investigation. When Nostoc ANTH was grown in BG-11₀ medium (N₂-medium), no akinete formation was evident at any stage of the growth. Akinete differentiation was not triggered even after altering the pH of the medium (from pH 7.5 to 5, 9, 11) or temperature at which the cells were cultured (20°, 25°, 30°, 35°, 45° or 50° C). Even when Nostoc
ANTH, grown in NH$_4^+$- or NO$_3^-$-supplemented BG-11$_0$ medium (NH$_4^+$- and NO$_3^-$-media, respectively), was transferred to BG-11$_0$ medium (N$_2$-medium), akinete formation was not triggered. Thus, limitation of combined nitrogen did not cause akinete differentiation (Table 1). Similarly, addition of glucose, fructose or sucrose (each at concentration of 10, 20, 30, and 50 mM) to the growth medium was ineffective in triggering akinete formation although such additions prolonged the exponential growth phase (Table 1). However, when N$_2$-grown cultures of Nostoc ANTH were transferred to fresh N$_2$-medium from which sulphate was omitted (BG-11$_0$ minus MgSO$_4$), there was no growth and akinete differentiation started within 3 d of the transfer and by day 24, all vegetative cells became akinetes (Table 1, Fig. 2).

Akinete differentiation also occurred when Nostoc ANTH from N$_2$-medium was transferred to N$_2$-medium lacking phosphate (K$_2$HPO$_4$). However, the akinete differentiation was delayed compared to that in the medium lacking sulphate (akinete differentiation started after 8 d of transfer instead of just 3 d). In addition, the akinetes continued to differentiate for a longer period than that in the medium lacking sulphate (35 d instead of 24 d). In the medium lacking phosphate, Nostoc ANTH also showed significant level of growth during the initial 7-8 d in contrast to the sulphate lacking medium where no growth was observed (Fig. 2). The initial growth of Nostoc ANTH in the medium lacking phosphate can be explained by the fact that repeated subculturing of cyanobacteria in laboratories leads to accumulation of phosphate (polyphosphate bodies) that can be mobilised under phosphate limiting conditions$^2$. Therefore, under phosphate-limiting conditions they continue to grow as long as internal reserve of phosphate lasts. Thus, for the first 7-8 d the cells grew in phosphate-limiting medium using internal reserve phosphate, after which growth ceased and akinete formation started. No such reserve for sulphate are known in cyanobacteria, therefore, the effect of sulphate limitation is quicker on cessation of growth and triggering akinete formation. In fact Nostoc ANTH lost the ability to form akinetes in

![Fig. 1—Light micrograph of Nostoc ANTH. a, akinetes; b, same as 'a' under fluorescence (excitation 545-580 nm); c, a filaments showing heterocyst (H) and vegetative cells (V); d, same as 'c' under fluorescence. The absence of florescence indicates lack of phycobiliprotein in akinetes and heterocysts. Bar = 100μm](image)

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Observations</th>
<th>$^a$ Start of akinete differentiation (d)</th>
<th>$^b$ End of akinete differentiation (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG-11$_0$</td>
<td>No akinetes were observed during or at the end of exponential phase of growth</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>No akinetes were observed in culture grown at pH 5, 7.5, 9 or 11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Temperature</td>
<td>No akinete were observed in culture grown at 20, 25, 30, 35, 45, or 50$^\circ$C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BG-11$_0$ + exogenous C sources (glucose, fructose or sucrose; 10-50 mM)</td>
<td>Exogenous C sources prolonged the exponential phase, but no akinete were observed during or at the end of exponential phase of growth</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BG-11$_0$ minus sulphate</td>
<td>Cessation of growth followed by akinete differentiation</td>
<td>3±1</td>
<td>24±2</td>
</tr>
<tr>
<td>BG-11$_0$ minus phosphate</td>
<td>Akinete differentiation observed during the exponential growth phase</td>
<td>8±1</td>
<td>35±2</td>
</tr>
</tbody>
</table>

$^a$ Time when akinetes first appeared.

$^b$ Time when maximum numbers of cells became akinetes and no further akinete differentiation occurred.
response to phosphate limitation after 3 years of repeated culturing in BG-11\textsubscript{0} medium in our laboratory. However, even after 5 years of repeated culturing in BG-11\textsubscript{0} medium, the same cyanobacterium, Nostoc ANTH retained the ability to differentiate akinetes in response to sulphate limitation. In medium lacking phosphate and sulphate, the growth and akinete formation response of Nostoc ANTH remained similar to that observed in the medium lacking only the sulphate.

An interesting phenomenon was noticed when these experiments were repeated using medium lacking sulphate that was buffered with 5 mM HEPES. As discussed above, there was no growth and akinete differentiated in BG-11\textsubscript{0} medium lacking sulphate. In contrast, Nostoc ANTH grew and no akinete differentiation occurred when the medium lacking sulphate was buffered with HEPES. Similarly, in nitrate-supplement BG-11\textsubscript{0} medium lacking sulphate, there was no growth and akinete differentiation occurred. However, when this medium was buffered with HEPES, there was growth and no akinete differentiation occurred. HEPES contains sulphonic acid and Nostoc ANTH may be using it as a source for S, at least under the sulphate limiting conditions, as indicated by the growth of Nostoc ANTH in HEPES-buffered media lacking sulphate. These results clearly show that the cessation of growth and triggering of akinete differentiation was due to sulphate limitation and that the addition of HEPES relieved this limitation. When in symbiosis with Anthoceros, Nostoc ANTH rarely forms akinetes although the growth rate is much slower in symbiosis than when free living. It is unclear what prevents akinete formation by the cyanobiont when in symbiosis and this aspect would merit further study.

Some of the above observations on akinete differentiation in Nostoc ANTH are consistent with the observations on akinete differentiation in other cyanobacteria by earlier workers. However, a number of features regarding akinete differentiation in Nostoc ANTH are unique and/or in contrast to the features of akinete differentiation in other cyanobacteria. Limitation of light due to the increase in culture density during growth that results in self shading, has been suggested as a trigger for akinete development\textsuperscript{11,12,14,26-28}. Furthermore, there have been reports that iron limitation\textsuperscript{29}, limitation of fixed nitrogen\textsuperscript{30,31} increase in concentration of NaCl\textsuperscript{32}, and provision of amino acids trigger or increase akinete differentiation in various cyanobacteria. However, the results of the present study indicate that this is not true in the case of Nostoc ANTH. As reported for Nostoc PCC 7524\textsuperscript{27}, addition of exogenous sources of fixed carbon prolonged the growth phase of Nostoc ANTH, but in contrast to the Nostoc PCC 7524, no akinetes were formed in Nostoc ANTH. The akinete formation in Nostoc ANTH under phosphate limitation is consistent with earlier reports implicating lack of phosphate as a major trigger of akinete development\textsuperscript{11,12,14,16}.

The triggering of akinete differentiation in Nostoc ANTH under sulphate limitation reported in the present investigation is the first report of its kind, and is in contrast to the report by Sinclair and Whithon\textsuperscript{29} that sulphate limitation has no effect on akinete differentiation in Anabaena cylindrica. It appears that the two cyanobacteria respond differently to sulphate limitation. Both under phosphate limitation and sulphate limitation, the akinete differentiation was associated with cessation of growth of Nostoc ANTH. This is consistent with similar observations on A. cylindrica\textsuperscript{25,33,34} Nostoc PCC 7524\textsuperscript{27} and Anabaena doliiolum\textsuperscript{26}. Overall, the data indicate that sulphate limitation is a powerful trigger, and better than phosphate limitation, for quicker and more profuse akinete formation in Nostoc ANTH.

Certain physiological properties of the akinete population was checked to compare with the earlier studies. The mature akinetes of Nostoc ANTH showed a respiratory O\textsubscript{2} consumption rate of 17.6% of that in N\textsubscript{2}-grown filaments but lacked photosynthetic pigments and O\textsubscript{2} evolution. These observations are
consistent with the findings regarding akinetes of *A. cylindrica*[^25,36], *Nostoc PCC 7524*[^27,37], *A. doliiolum*[^35,38] and *Nostoc spongiateforme*[^39]. The mature akinetes of *Nostoc ANTH* also lacked the primary enzymes of inorganic nitrogen metabolism such as nitrogenase, nitrate reductase and glutamine synthetase (Table 2). The lack of these enzymes in mature akinete of *Nostoc ANTH* is also in keeping with the finding on akinete of *A. doliiolum*[^38] and of other cyanobacteria[^40].

To check that these akinetes were viable, we harvested akinetes from sulphate-limiting medium (BG-11o lacking sulphate), resuspended them in fresh N₂-medium (BG-11o) and incubated in light at 25°C. About 25% of the akinetes, in suspension, germinated within 24 h and a germination frequency of 95% was achieved after 96 h. The akinetes showed prolonged viability. Prolonged storage of akinetes in dry state at room temperature did not lead to any significant loss of viability. Over 90% of the akinetes germinated even after 4-5 years of storage at room temperature. When stored at higher temperatures (upto 35°C) or at 4°C, there was still no loss in viability of the akinetes and > 90% of akinetes germinated, even after 5 years of storage.

In summary, the results presented in this investigation clearly showed that sulphate limitation can be used as a trigger to induce quick and profuse akinete formation in *Nostoc ANTH*. The excellent germination rate, even after long-term storage at room temperature, makes these akinetes a good candidate for use as inoculum if *Nostoc ANTH* is used as biofertiliser in rice paddies.

**References**

8. Singh R N, Role of blue-green algae in nitrogen economy of *Indian agriculture* (Indian Council of Agricultural Research, New Delhi, India) 1961.

**Table 2—Activities of nitrogen-metabolising enzymes in whole filaments and akinetes of *Nostoc ANTH***

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (nmol product formed min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Akinetes</td>
</tr>
<tr>
<td>Nitrogenase</td>
<td>0.0</td>
</tr>
<tr>
<td>Glutamine synthetase(transferase)</td>
<td>0.0</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*nmol C₅H₆O₄ formed g⁻¹ Chlorophyll a.h⁻¹
The values presented are means ± SE of two independent experiments.
31 Dementer O, Über modificationen bei cyanophyceen, Arch Mikrobiol, 24 (1956) 105-133.