Methylammonium Uptake in the Thermophilic Cyanobacterium, *Mastigocladus Laminosus*

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ABSTRACT

The ammonium transport system of thermophilic cyanobacterium, Mastigocladus laminosus was studied as a function of temperature and nitrogen sources using [^{14}C]-methylammonium, an analogue for ammonium. Ammonium transport activity was found to be 14 % higher in dinitrogen-grown cultures at 45°C than at 25°C grown cultures. The uptake was found to be biphasic, consisting of an initial fast phase lasting upto 60 sec, followed by a slower second phase. The ammonium transport activity was repressed in nitrate- and ammonium-grown cultures.

Introduction

Cyanobacteria are known to utilize inorganic and organic nitrogen compounds as nitrogen sources (Bhattacharya *et al.*, 2002*a*). Among inorganic sources of nitrogen, ammonia is the preferred nitrogen source and it is assimilated by the GS-GOGAT pathway (Flores & Herrero, 1994). Ammonia is produced in the heterocysts of diazotrophic cyanobacteria mainly by N_2-fixation (Herrero *et al.*, 2001). The activity and synthesis of nitrogenase enzyme is repressed in the presence of ammonia in the growth medium. While exogenous NH_4^+ requires to be transported into the cell through a transport system, NH_3 diffuses across the membrane and can be trapped in the cell by protonation (Kleiner, 1981). [^{14}C]-methylammonium, an analogue of ammonium, can be used as a probe to study the ammonium transport system in cyanobacteria (Rai *et al.*, 1984). Ammonium transport systems have been studied extensively in many N_2-fixing mesophilic cyanobacteria (Boussiba *et al.*, 1984; Rai *et al.*, 1984, Singh *et al.*, 1985, Vega-Palas *et al.*, 1990, Prakasham & Rai, 1991; Singh *et al.*, 1991; Bhattacharya *et al.*, 2000*a*). However the

information regarding activity of ammonium transport system in the thermophilic cyanobacteria is not available and deserves attention.

We have isolated and purified a thermophilic cyanobacterium *Mastigocladus laminosus* from the hotspring at Jakrem (Meghalaya, India). *M. laminosus* is a cosmopolitan thermophilic cyanobacterium found in thermal waters on every continent (Castenholz, 1969). It is also the most thermophilic nitrogen-fixing cyanobacterium with an upper temperature limit for nitrogen-fixation of up to 60°C (Stewart, 1970). This species is ecologically important as a component of algal bacterial mats in neutral to alkaline thermal springs. *M. laminosus* has been investigated from an ecological standpoint (Castenholz, 1976, 1977; Fagerberg & Arnott 1979) and for its potential for biophotolysis of water (Miyamoto *et al.*a,b; 1979; Miura *et al.*, 1980). We have studied the effect of N_2 NO_3^-, NH_4^+ on growth, heterocyst frequency, nitrogenase activity and methylammonium uptake activity in this cyanobacteria. Our results suggest that nitrate is the best source of utilizable nitrogen for growth of this cyanobacterium followed by NH_4^+ and N_2, and that nitrogenase, heterocyst formation and ammonium transport activity are nitrogen source repressible. Furthermore, growth, nitrogenase activity, heterocyst formation and ammonium transport activity were higher at 45°C than that at 25°C.

Materials and Methods

1. Strains and culture conditions

The thermophilic cyanobacterium *Mastigocladus laminosus* was maintained on agar slants as well as in liquid D-medium (Castenholz, 1981). Cultures were maintained at 25°C (culture room) or 45°C (inside a B.O.D. incubator) and light was provided at a photon fluence rate of 50 μ mol $\cdot$ $m^{-2} \cdot s^{-1}$ on the surface of the vessels. Dinitrogen medium refers to medium D without any N ($NaNO_3$ and KNO_3 replaced by NaCl and KCl, respectively), nitrate medium to medium D (10mM $NaNO_3$) and ammonium medium to 2 mM NH_4 Cl added to the D – N_2 medium. The NO_3^- or NH_4^+ supplemented medium was buffered with equimolar concentration of HEPES and pH was adjusted to 7.5 before autoclaving.

2. Growth, heterocyst frequency and nitrogenase activity

Growth (in terms of increase in Chlorophyll *a*) was measured using the method of Mackinney (1941). Heterocyst frequency was calculated as percentage of total cell population by light microscope. Nitrogenase activity was measured using acetylene reduction assay (Stewart *et al.*, 1967)

3. Ammonium transport assay

Ammonium uptake by *Mastigocladus laminosus* was studied using ^{14}C-methylammonium, an analogue of ammonium (Rai *et al.*, 1984). Exponentially growing cells of *M. laminosus* were harvested, washed and resuspended in fresh $D-N_2$-medium, fresh NO_3^- -medium or NH_4^+-medium and then incubated at 25°C or at 45°C for 48 h. The cells were then harvested, washed and resuspended in 10 mM HEPES-NaOH buffer, pH 7.0, to a concentration of 10 $\mu g\ Chl\ a \cdot ml^{-1}$ and equilibrated for 1 hr at 45 °C or 25 °C under light (photon fluence rate: 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) inside a B.O.D. incubator. The ^{14}C labeled methylammonium uptake experiment was iniated by adding ^{14}C methylammonium at a final concentration of 50 µM (specific activity 350 KBq. μmol^{-1}). At different time intervals, 400 µl samples were taken out rapidly and the cells were separated from their bathing medium by microcentrifugation through silicon oil/dinonyl phthalate (45/55, v/v) into perchloric acid/water (15/85, v/v) as described previously (Scott & Nicholls, 1980; Rai *et al.*, 1984). The amount of ^{14}C in perchloric acid fraction was determined by using a liquid scintillation counter (Model 1801, Beckman Instruments). Non-specific binding of radioactivity was determined by measuring its incorporation in the toluene-treated cells as described by Rai *et al.* (1984). These values were always substracted from the values obtained for untreated samples.

Results and Discussion

1. Growth, heterocyst and nitrogen fixation.

The growth, heterocyst frequency and nitrogenase activity of *Mastigocladus laminosus* were measured at 25 °C and 45 °C in N_2, and NH_4^+ supplemented medium. *M. laminosus* was able to utilize

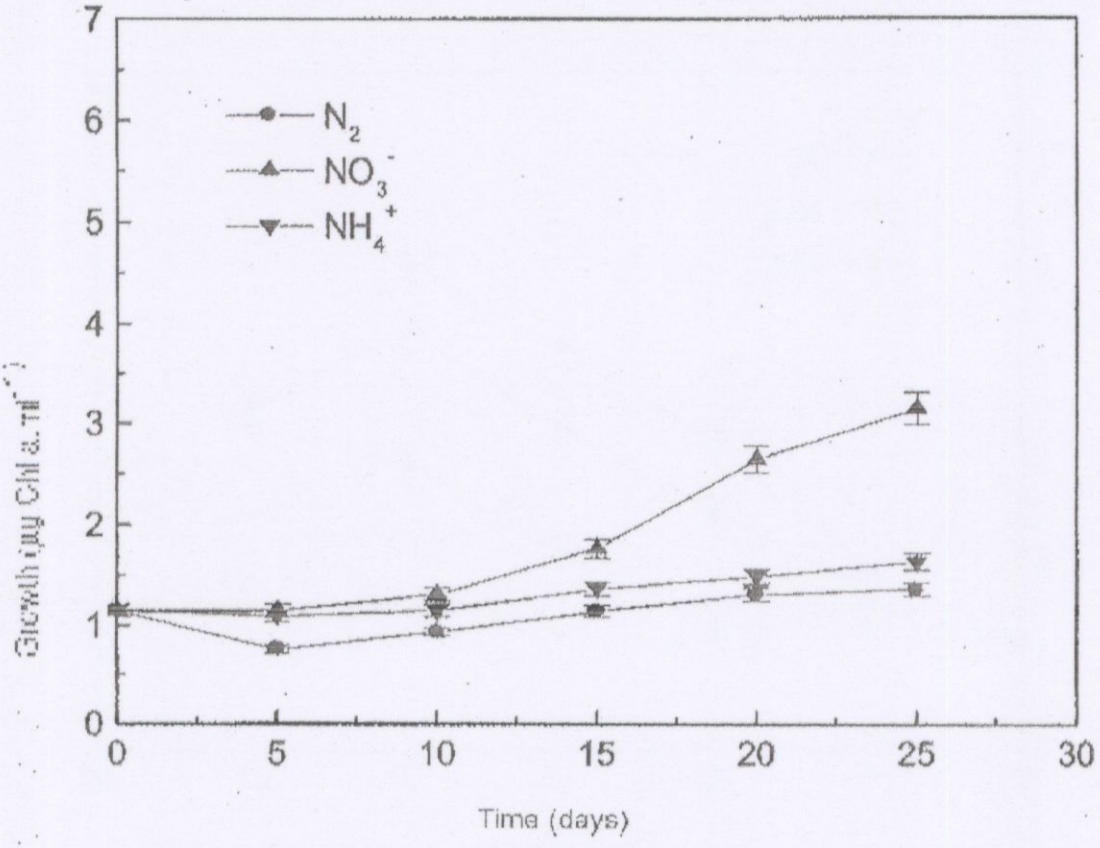

Fig. 1. Growth of ***Mastigocladus laminosus*** in media containing different nitrogen sources at 25 °C. Growth in terms of increase in Chl ($\mu g \cdot ml^{-1}$ was determined after intervals of five days. The initial inoculum concentration was 1.1 μg Chl a · ml– 1 · Nsub mediuum (↑), NO_3^{-1} medium (σ) and NH_4^+ medium (σ)

these inorganic nitrogen sources for growth as is the case with other heterocystous cyanobacteria (Herrero & Flores, 1990; Flores & Herrero, 1994; Herrero *et al.*, 2001; Bhattacharya *et al.*, 2002*a*) at 25 °C (Fig 1). As shown in Fig 1 & 2, nitrate served as the best source of nitrogen for growth (measured as increase in Chl *a*), followed by ammonium and then N_2. However, unlike most mesophilic heterocystous cyanobacteria, *M. laminosus* grew also at a temperature of 45 °C (Fig 2). While the trend of relative growth performance in different nitrogen-media remained similar to that at 25°C, the growth was significantly higher at 45 °C (Fig. 1, 2).

In N_2-medium, *M. laminosus* differentiated heterocysts at a frequency of 10 and .3 % at 45°C and 25°C respectively. Similarly

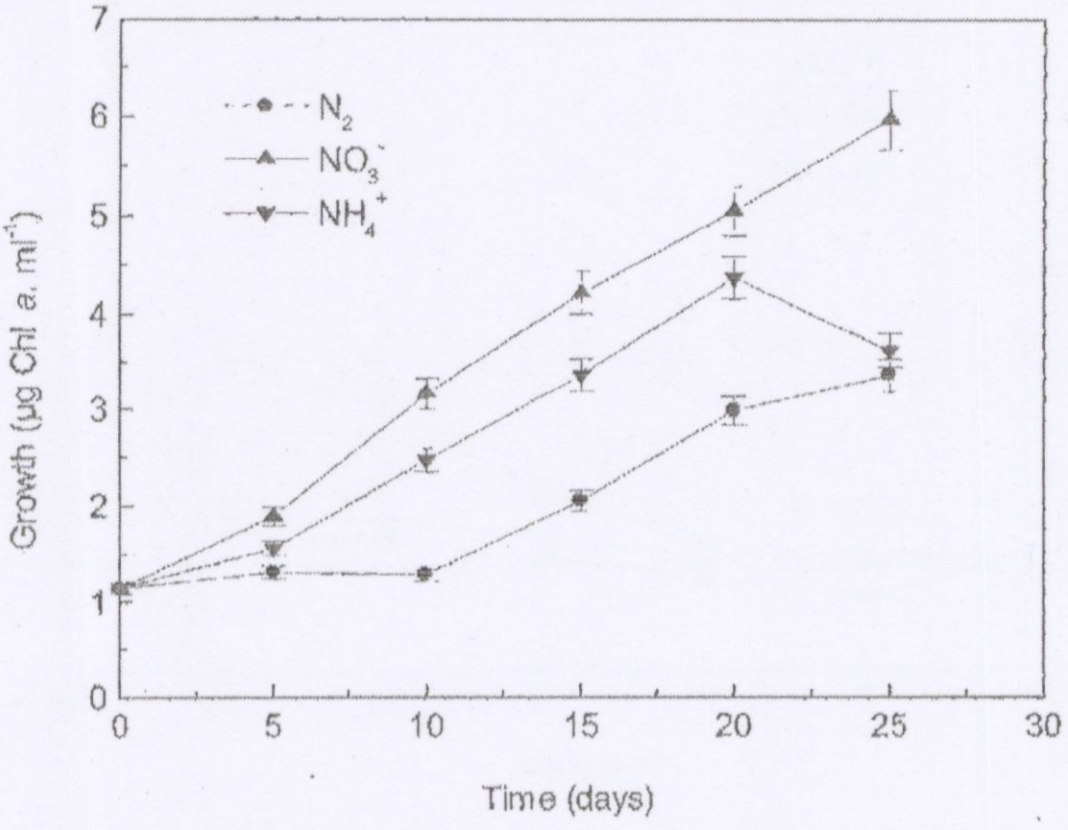

Fig. 2. Growth of ***Mastigocladus laminosus*** in media containing different nitrogen sources at 45 °C. Growth in terms of increase in Chl *a* ($\mu g \cdot ml^{-1}$) was determined after intervals of five days. The initial inoculum concentration was 1.1 µg Chl a · ml– 1 · Nsub mediuum (↑), NO_3^{-1} medium (σ).

the level of nitrogenase activity at 45°C was two fold higher (4.2 nmol of C_2H_4 formed · µg Chl $a^{-11} \cdot hr^{-1}$)

Heterocyst frequency (%) and nitrogenase activity (nmol of C_2H_4 formed. µg Chl $a^{-1} \cdot hr^{-1}$ were determined after 4 days of inoculation to the fresh medium-D with and without nitrogen sources (Castenholz, 1981). The initial inoculum concentration was 1.5 µg Chl a · ml^{-1}. The values presented are means± standard error (SE) from two independent experiments, each with two replicates. N2 refers to medium D without any N, NO_3^{-3} to medium D (10 mM $NaNO_3$) and NH_4^+4+ to medium D with NH_4Cl (2 mM) as N source

than at 25°C (2.0 nmol of $C_{2\,4}$ formed · μg Chl a^{-1} · hr^{-1}). (Table 1). Both heterocyst frequency and nitrogenase activity were fully repressed under nitrogen source supplemented medium at both temperatures (45°C and 25°C). These observations are consistent with reported growth of *M. laminosus* in hotsprings with temperatures varying between 45-60°C (Binder *et al.*, 1972) and the repressive effect of NO_3^- and NH_4^+ on heterocyst formation and nitrogense in heterocystous cyanobacteria (see Stewart, 1980; Bhattacharya *et al.*, 2002*a*).

Table 1: Heterocyst frequency and nitrogenase activity of ***Mastigocladus laminosus*** **cells grown in media containing different nitrogen sources at 25°C and 45°C.**

Medium	Heterocyst frequency (%) 45°C	25°C	Nitrogenase activity 45°C	25°C
N_2	10 ± 0.5,	3 ± 0.2	4.2 ± 0.2	2 ± 0.1
NO_3^-	0.0	0.0	0.0	0.0
NH_4^+	0.0	0.0	0.0	0.0

2. Methlammonium transport system.

^{14}C-methylammonium (an analog of ammonium) was used as a probe for studying methylammonium transport activity in *M. laminosus*. The results of methylammonium uptake experiments in *M. laminosus* are shown in Fig 3. N_2-grown cultures showed biphasic pattern of methylammonium uptake kinetics consisting of an initial fast phase lasting 60 sec, followed by a slower second phase. This trend was observed both at 45°C and 25°C. The methylammonium uptake rates in N_2-grown cultures during the first and second phase at 45°C were 42 and 17.55 nmol. mg Chl a^{-1}. min^{-1} and at 25°C, 36 and 3.7 nmol. mg Chla^{-1} · min^{-1}, respectively. The biphasic nature of methylammonium uptake in N_2-grown cultures was in total agreement with that observed earlier in other heterocystous cyanobacteria (Rai *et al.*, 1984; Singh *et al.*, 1985; Boussiba *et al.*, 1984; Bhattacharya *et al.*, 2002*b*). The methylammonium uptake

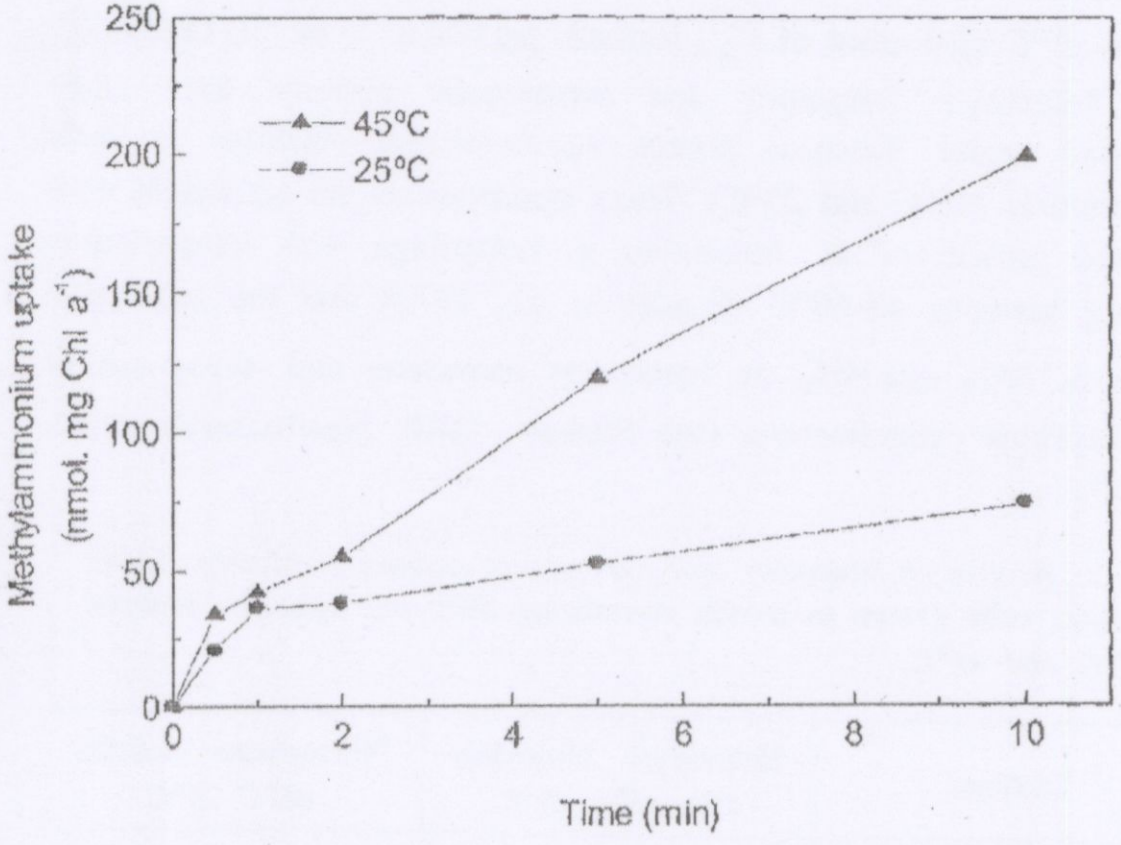

Fig. 3. [^{14}C]-methylammonium uptake in *Mastigocladus laminosus* grown in N_2-medium (25 °C, ↑; 45 °C, *symbnls*). Nitrate–grown cultures (exponential phase) were washed and transferred to N_2-medium media and incubated for 48 hr. The cultures were then harvested, washed and resuspended in HEPES buffer, and used for [^{14}C]-methylammonium uptake as described in Materials and Methods. The values presented are means from two independent experiments, each with two replicates.

system was found to be repressed in NO_3^- and NH_4^+ grown cells at both temperatures. This observation was in consistent with earlier reports in other cyanobacteria (Prakasham & Rai, 1991; Singh *et al.*, 1991; Bhattacharya *et al.*, 2002*a*). The higher ammonium (methylammonium) transport activity noted in *M. laminosus* at elevated temperatures is in contrast to the mesophilic cyanobacteria and in keeping with the thermophilic nature of *M. laminosus.*

Thus the overall results showed that utilization of inorganic nitrogen sources, formation of heterocysts, nitrogenase activity and ammonium transport activity in the thermophilic cyanobacterium, *M. laminosus* were higher at 45°C than those at 25°C.

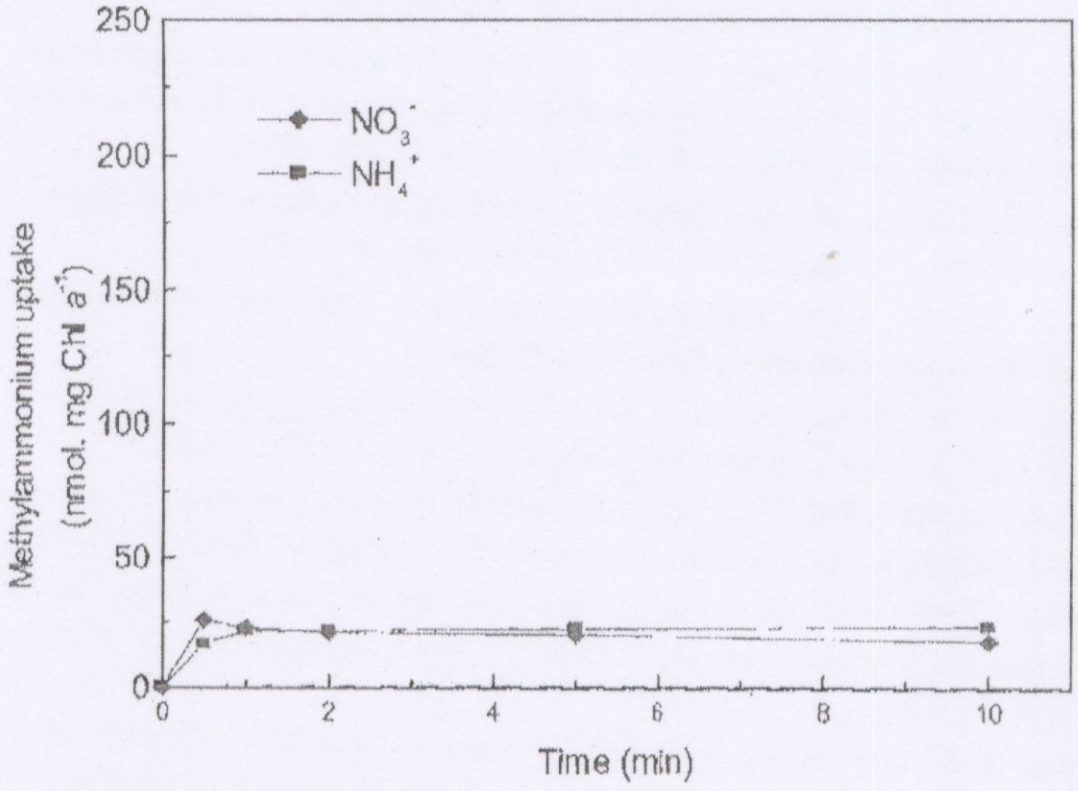

Fig. 4. [^{14}C]-methylammonium uptake in *Mastigocladus laminosus* grown at 25 °C and 45 °C in NO_3^--medium (υ), and NH^+ and NH^{+4}-medium (ν). Nitrate–grown cultures (exponential phase) were washed and transferred to NO_3^- – and NH^{+4}-medium and incubated for 48 hr. The cultures were then harvested, washed and resuspended in HEPES buffer, and used for [^{14}C]-methylammonium uptake as described in Materials and Methods. The values presented are means from two independent experiments, each with two replicates. Similar values were obtained for experiments carried out at 25°C and 45°C.

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