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Ammonium Excretion by a Mutant of the Nitrogen-Fixing Cyanobacterium *Anabaena siamensis*

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Abstract

Anabaena siamensis *isolated from rice fields in Thailand is a fast growing cyanobacterium with a high nitrogen-fixing activity. Mutant strains resistant to the* L*-glutamate analogue,* L*-methionine sulfoximine (MSX) were isolated by ethyl methanesulfonate mutagenesis. A stable mutant named* A. siamensis *SS1, which released ammonium to the medium, was studied further. In batch cultures the rate of ammonium production peaked at the early log phase and gradually decreased until the 4th day of growth when the cultures reached a density of 90 μg chl ml^{-1}. To obtain constant release of ammonium by SS1, continuous culture experiments were performed at a cell density of 5 μg chl ml^{-1} and the following results were obtained: (1) growth rate as the parent (μ:0·123 h^{-1}) in the presence and absence of 500 μM MSX; (2) 48% GS transferase activity when compared with the parent; (3) ammonium excretion at a rate of 8 μmol (mg chl)$^{-1}$ h^{-1} as measured up to 20 generations (120 h); (4) derepressed nitrogenase activity; and (5) 30% higher nitrogenase activity than that of the parent. SS1 immobilized in alginate beads (5 μg chl ml^{-1}) exhibited values of glutamine synthetase and nitrogenase activity similar to those of free cells. However, ammonium excretion at the rate of 11·61 μmol (mg chl)$^{-1}$ h^{-1} was obtained only up to 20 h after loading in bioreactors, due to the fast growth of SS1 as also occurred in batch cultures.*

Key words: Anabaena siamensis, mutagenesis, amino acid analogue resistance, nitrogen fixation, ammonium excretion, glutamine synthetase, rice fields.

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INTRODUCTION

The heterocystous cyanobacteria can utilize light energy to support both carbon dioxide fixation and nitrogen fixation under aerobic conditions (Stewart, 1980). This allows them to produce fertilizer nitrogen that can contribute to the growth of plants. The agronomic significance of cyanobacteria, either free-living or in symbiotic association with the aquatic fern, *Azolla*, has long been recognized (Moore, 1969; Venkataraman, 1975). The application of nitrogen-fixing cyanobacteria in the Orient replaces 20–30% of the chemical nitrogen fertilizer demand, increasing the natural fertility of the paddy-field soils (Venkataraman, 1975).

However, the inoculation of non-indigenous cyanobacterial strains is found to be not totally successful due to the failures to overcome the interspecific competitions and environmental constraints (Grant *et al.*, 1985). There is hence a need to identify ideal strains from the indigenous population of a particular location of interest for the production of inoculum for algalization, and to assess its effects upon rice yields. In this respect, *Anabaena siamensis* isolated from Thai rice fields appears to be a promising organism for algalization, due to its fast growth rate and adaptation to wide temperature (25–42°C) and salinity (1–2%) ranges (Antarikanonda, 1982*a*; Antarikanonda & Lorenzen, 1982).

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The nitrogen fixed by cyanobacteria is made available to the environment mainly by autolysis and mineralization after death (Martinez, 1984). Free-living cyanobacteria release ammonium in insignificant quantities during growth but can excrete it in high amounts when treated with MSX, a highly specific, irreversible inhibitor of glutamine synthetase (Ronzio *et al.*, 1969). However, in symbiotic association, cyanobacteria such as *A. azollae* and *Nostoc* sp. 7801, release fixed nitrogen to the host's nitrogen requirements in the form of ammonium due to an inhibition of GS activity under symbiotic conditions (Orr & Haselkorn, 1982; Joseph & Meeks, 1987). Ammonium excretion in cyanobacteria seems to be dependent upon GS rather than on nitrogenase activity. Actually, mutations in the GS structural gene, *gln*A, causing reduction of GS activity result in excretion of ammonium (Polukhina *et al.*, 1982; Hien *et al.*, 1988). Based on this principle, an ammonium excreting mutant of *A. variabilis* has been found to be effective as a supplier of nitrogen fertilizer to rice plants in laboratory experiments (Lattore *et al.*, 1986). However, growth of these strains can be very slow when compared to their parent strains, which in turn are not indigenous organisms. Hence, we have selected a rice field isolate (*A. siamensis*) which is already marketed as algal biofertilizer for rice crops and claimed to increase the growth and yield of rice plants due to its high nitrogen-fixing capacity (Antarikanonda, 1982*b*, *c*).

In this paper we describe the isolation and characterization of an ammonium excreting mutant of *A. siamensis*.

METHODS

Organism and growth medium

The nitrogen-fixing cyanobacterium, *Anabaena siamensis* used in this study is an isolate from Thailand (Antarikanonda, 1985) and has been deposited in the Sammlung von Algenkulturen, Pflanzenphysiologisches Institute, Universität Göttigen, Federal Republic of Germany under the signature *Anabaena* spec. B 11.82. The components of growth medium (Antarikanonda, 1982*a*) (AS medium) contained per liter: 0·20 g $K_2HPO_4.3H_2O$, 0·25 g $MgSO_4.7H_2O$, 0·056 g $CaCl_2$, 0·23 g NaCl, 0·025 g $FeSO_4.7H_2O$, 0·027 g Titriplex III, 0·002 g $MnCl_2.4H_2O$, 0·0015 g $NaMoO_4.2H_2O$, 0·0002 g $ZnSO_4$, 0·00008 g $CuSO_4.5H_2O$, 0·00002 g $CoCl_2.6H_2O$ and 0·003 g H_3BO_3. After autoclaving the pH was adjusted to pH 7·5 and the phosphate solution was autoclaved separately.

Growth conditions

A. siamensis was cultivated in 500-ml sterilized glass columns placed in a transparent plexiglass circulating water bath. The water temperature was maintained at 42°C. A constant photon flux of 175 μE m^{-2} s^{-1} at the surface of the growth vessel was supplied laterally by a battery of eight cool-white fluorescent lamps. Continuous aeration was provided by bubbling filtered air containing 1·5% CO_2. The pH was thus maintained at 7·0–7·2. Unless otherwise stated, cultures were sampled during the logarithmic growth phase for use in the different experiments.

Continuous culture experiments were carried out as described previously (Boussiba & Richmond, 1980). The cultures were maintained at 5 μg chl ml^{-1}. Cells were harvested up to 20 generations (120 h) and used for different experiments.

Immobilization of cyanobacteria was carried out by using the alginate entrapment method (Kerby *et al.*, 1986) with a few modifications. Cyanobacterial cell suspensions at 10 μg ml^{-1} concentration of chlorophyll were mixed with equal volumes of 3% sodium alginate solution and dropped in 0·1 M $CaCl_2$ solution through a capillary tube to form beads of 2–5 mm diameter. The alginate-entrapped cyanobacteria were collected after 2 h and kept at 4°C for 12 h. The beads were loaded in packed-bed reactors. The flow rate of the culture medium was 0·2 h^{-1}.

Growth determination

Growth was followed by means of chlorophyll determination, starting from an initial inoculum containing 1 μg chl ml^{-1}. Chlorophyll *a* was determined colorimetrically in methanol extracts (MacKinney, 1941). Protein was determined colorimetrically after digestion with 0·5 N NaOH by Lowry's method (Lowry *et al.*, 1951).

Mutagenesis

Mutagenesis with ethyl methanesulfonate was carried out as reported by Spiller *et al.* (1986) with a few modifications. Mutants resistant to L-methionine-DL-sulfoximine (MSX) were selected on agar plates containing 500 μM MSX. Ammonium-excreting mutants were selected on solid media containing the pH indicator phenol red. One of the mutants, which induced the most marked

colour change in the plates (from red to pink), was used for further studies and designated as *A. siamensis* SS1.

A filament of *A. siamensis* generally consists of 8–10 vegetative cells between two terminal heterocysts. Before treating with the mutagen the filament length was reduced to 1–3 cells by providing mild sonication for 3 s in a sonicator bath.

Ammonium uptake

Ammonium uptake was followed for 20 min starting from an initial concentration of 100 μM, as described previously (Zimmerman & Boussiba, 1987).

Ammonium determination

Filtrates from cyanobacterial cultures were collected during growth at different time intervals to estimate the ammonium released into the medium. Ammonium concentration in the medium was measured by Solorzano's phenol-hypochlorite method (Solorzano, 1969).

Enzyme assays

Nitrogenase activity was estimated in intact filaments by the acetylene reduction assay (ARA) (Stewart *et al.*, 1967). Samples of 4·6 ml of cyanobacterial culture were washed in fresh AS medium and placed in a 25-ml Wheaton bottle sealed with a flanged rubber septum. The Wheaton bottles were placed on a rotary shaker (100 rpm) while being illuminated with a quantum flux of 75 μE m^{-2} s^{-1} during the assay. The filaments were allowed to stand for 10 min before injection of acetylene. Ethylene was analyzed with an HP 5890 gas chromatograph (California, USA) using a stainless steel column packed with Porapack-N (0·2 cm id, 265 cm length). The nitrogenase activity was expressed as μmol C_2H_4 produced $(mg\ chl)^{-1}\ h^{-1}$.

Nitrogenase activity in immobilized cyanobacteria was determined by incubating the alginate beads containing cyanobacteria (= 5 μg chl ml^{-1}) in 130-ml Wheaton bottles and assayed as described above.

Glutamine synthetase (GS) was assayed in concentrated suspensions (1 mg protein ml^{-1}). Cells were permeabilized with 2% toluene for 1 min and kept in ice for 15 min before the activity measurements. Activity was measured as transferase (Sampio *et al.*, 1979) and expressed in μmol γ-glutamyl hydroxamate formed $(mg\ protein)^{-1}\ min^{-1}$.

Chemicals

L-methionine-DL-sulfoximine (MSX) was purchased from Sigma Chemical Co., (St Louis, Missouri, USA) and other chemicals were from E. Merck AG, (Darmstadt, FRG). Sodium alginate was the product of Aldrich Chemical Co., USA.

RESULTS AND DISCUSSION

Under our standard conditions, *A. siamensis* exhibited a high growth rate (doubling time 5·6 h), higher than other rice-field nitrogen-fixing cyanobacterial strains reported in the literature. The nitrogenase activity was also high (350 nmol $(mg\ protein)^{-1}\ min^{-1}$). This strain has been reported to release amino acids to the environment (Antarikanonda, 1984). However, it did not excrete ammonium during active growth. On the other hand, if the GS inhibitor, L-methionine sulfoximine (MSX) was added to the cell suspensions, ammonium was released into the medium in significant quantities (data not shown). MSX concentrations above 100 μM inhibited the growth of *A. siamensis* significantly and 500 μM MSX was completely lethal in liquid as well as solid media (Fig. 1). Among the MSX-resistant strains selected, only a few were able to change the color of phenol red, orange to pink, in pH indicator plates. Another class of mutants grew well in the presence of 500 μM MSX, apparently because of an MSX-resistant GS, but they did not induce color change in pH indicator plates. A third class of MSX-resistant mutants were those which survived at 500 μM MSX in a solid medium, but not in a liquid medium. A fourth group comprised the MSX-resistant mutants which tolerated high concentrations (1000–1500 μM). Among the mutants belonging to the first group, a particular strain (designated SS1), which *promoted* a very marked change in the color of phenol red, was selected for a detailed study.

A. siamensis SS1 grew as the parent strain under all growth conditions tested (Table 1). In batch cultures the nitrogenase activity was high during the early log phase, with a maximum value (30% higher than the parent strain) after 12 h of inoculation, decreasing thereafter. The ammonium release followed a similar trend, being a maximum at 12 h and slowing down during the late log to be almost negligible at the stationary phase (Fig. 2). This behaviour can be explained, since when growth progressed the availability of light was progressively limited due to the high

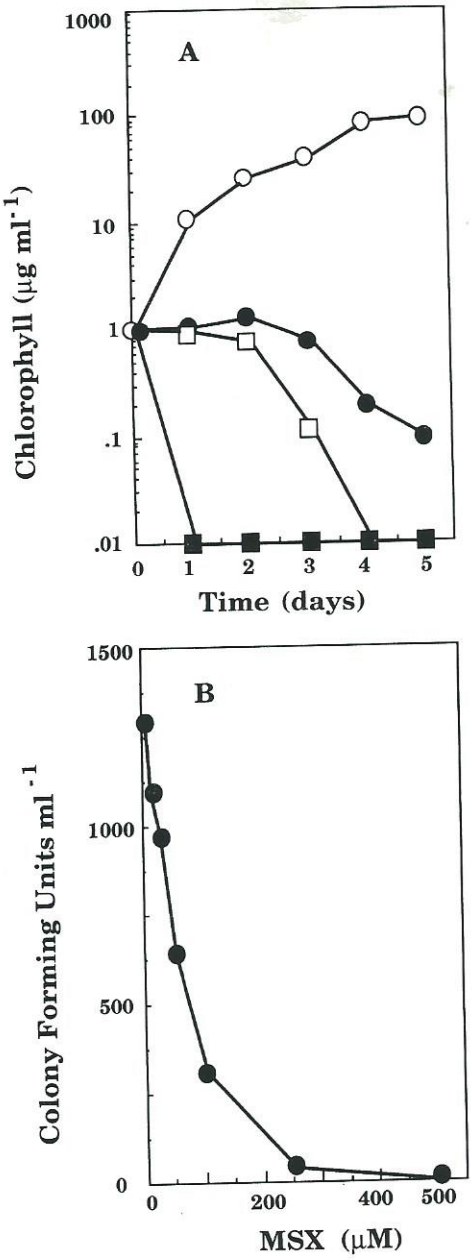

Fig. 1. Influence of MSX on the growth of *Anabaena siamensis* in liquid (A) (○—○, control; ●—●, 100 μM MSX; □—□, 250 μM MSX, and ■—■, 500 μM MSX) and solid (B) media.

levels of pigmentation, and nitrogen fixation was therefore lowered. In reflection of this, the rate of ammonium release was reduced significantly. In addition, part of the released ammonium was probably taken up by the cells since SS1 showed the same ammonium uptake rate in the presence (30·6 nmol (μg protein)$^{-1}$ min^{-1}) and absence (30·1 nmol (μg protein)$^{-1}$ min^{-1}) of MSX, at rate analogous to that of the parent in the absence of MSX (32·6 nmol (μg protein)$^{-1}$ min^{-1}). In order to improve ammonium release by SS1 the culture was diluted to 5 μg chlorophyll ml^{-1} and maintained in continuous culture. High ammonium excretion was observed for a prolonged period with a high nitrogenase activity. Immobilization of cells in alginate beads increased nitrogen fixation by 50% over that of the parent and increased the rate of ammonium excretion by 33% over the cell-free system. However, under the immobilized system ammonium release occurred only up to 20 h after loading in packed-bed reactors, when the initial cell concentration was of 5 μg chloro-

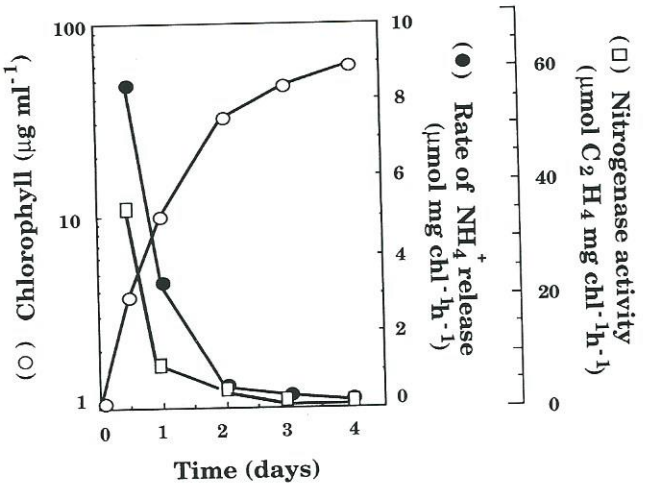

Fig. 2. Growth, nitrogenase activity and rate of ammonium excretion in batch cultures of *A. siamensis* mutant strain SS1.

Table 1. Ammonium release, growth rates and GS and nitrogenase activities for the parent strain of *A. siamensis* and the mutant SS1, under different growth conditions

Physiological pardmeter	*Batch culture*		*Continuous culture*		*Immobilized cells*	
	Parent	*SS1*	*Parent*	*SS1*	*Parent*	*SS1*
μ (h^{-1})	0·123	0·123	0·123	0·123	0·123	0·123
Nitrogenase activity (μmol C_2H_4 (mg chl)$^{-1}$ h^{-1})	21·0	35·5[a]	24·5	35·3	20·5	41·3
GS activity (μmol γ-glu. (mg protein^{-1} min^{-1})	3·6	1·3	3·0	1·4	3·1	1·2
Rate of NH_4^+ excretion (μmol (mg chl)$^{-1}$ h^{-1})	0·0	8·7*	0·0	8·7	0·0	11·6

[a]Measured after 12 h of inoculation.

phyll ml^{-1}. The growth on alginate beads was similar to that in batch cultures and the time course of ammonium excretion under immobilized conditions being also analogous. However, under all the conditions assayed, glutamine synthetase activity of the strain SS1 was less than 50% of that of the parent (Table 1). In order to check whether the MSX resistance and the reduced GS were due to a failure in transport of MSX into the mutants cells, GS activity was assayed at 500 μM MSX in the assay mixture. In the presence of MSX the parent's GS activity was completely inhibited whereas that of SS1 remained unaffected. Based on the above results, it is concluded that maintaining SS1 at a low cell density under continuous cultivation is a suitable condition for sustained excretion of ammonium by the cells.

Ammonium-excreting mutants of cyanobacteria are useful for many purposes. Mutants of *A. variabilis* have been used to study the mechanisms of ammonium uptake and assimilation and to understand the regulation of enzymes involved in amino-acid biosynthesis and nitrate assimilation (Spiller *et al.*, 1986). The mutant SA1 of *A. variabilis* has been proved to be an effective nitrogen-fertilizer source to contribute to the growth of rice plants (Lattore *et al.*, 1986). The fast growth rate of SS1 represents an advantage for the production of inoculum for algalization on a short-time basis. Field studies of SS1 in rice fields, in comparison with the parent strain, will provide information on the usefulness of SS1 in increasing the growth and grain yield of rice.

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