APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1990, p. 3499–3504
0099-2240/90/113499-06$02.00/0

Vol. 56, No. 11

Ammonium Excretion by an L-Methionine-DL-Sulfoximine-Resistant Mutant of the Rice Field Cyanobacterium *Anabaena siamensis*

SELWIN P. THOMAS,[1] ARIEH ZARITSKY,[2] AND SAMMY BOUSSIBA[1]*

Microalgal Biotechnology Laboratory, The Jacob Blaustein Institute for Desert Research, Ben Gurion University of the Negev, Sede Boqer 84990,[1] and Department of Biology, Ben Gurion University of the Negev, Be'er-Sheva 84105,[2] Israel

Received 29 May 1990/Accepted 28 August 1990

An ammonium-excreting mutant (SS1) of the rice field nitrogen-fixing cyanobacterium *Anabaena siamensis* was isolated after ethyl methanesulfonate mutagenesis by selection on 500 μM L-methionine-DL-sulfoximine. SS1 grew in the presence and absence of L-methionine-DL-sulfoximine at a rate comparable to that of the wild-type strain, with a doubling time of 5.6 h. The rate of ammonium release by SS1 depended on cell density; it peaked at the 12th hour of growth with 8.7 μmol mg of chlorophyll^{-1} h^{-1} (at a chlorophyll concentration of 5 μg ml^{-1}) and slowed down to almost nil at the fourth day of growth. A similar pattern of release by immobilized SS1 was observed between 12 to 20 h after loading alginate beads in packed-bed reactors at the rate of 11.6 μmol mg of chlorophyll^{-1} h^{-1}. The rate was later reduced significantly due to the fast growth of SS1 on the substrate. Prolonged release of ammonium at the peak level was achieved only by maintaining SS1 under continuous cultivation at low chlorophyll levels (5 to 7 μg ml^{-1}). Under these conditions, nitrogen fixation in the mutant was 30% higher than that in its parent and glutamine synthetase activity was less by 50%. Immunoblot analysis revealed that SS1 and its parent have similar quantities of glutamine synthetase protein under ammonium excretion conditions. In addition, a protein with a molecular weight of about 30,000 seems to have been lost, as seen by electrophoretic separation of total proteins from SS1.

Nitrogen-fixing cyanobacteria are being used as nitrogen biofertilizers in rice fields in countries where rice is the major staple diet (30, 31). Although some strains which thrive in rice fields release small quantities of the major fertilizing product, ammonia, during active growth, most of the fixed products are made available mainly through autolysis and microbial decomposition (18). Under these circumstances, it is difficult to control the flow of nitrogen compounds needed for the development of rice plants. A possible solution to this problem is to develop strains of cyanobacteria which release ammonium continuously.

Mutants of *Anabaena variabilis* and *Nostoc muscorum* resistant to the ammonium analog ethylenediamine and to the L-glutamate analog L-methionine-DL-sulfoximine (MSX) have been reported to release ammonium (11, 12, 22, 24, 26). They exhibit, however, a slower growth rate as compared with that of their parents. Another major problem of using cyanobacteria as biofertilizers is the competition between indigenous and introduced strains, the former generally dominating. It was assumed that ammonium-excreting mutants that had been isolated from strains indigenous to rice fields would overcome the constraints of the rice field environment better than strains derived from other habitats. The reinoculation and establishment of these mutants in rice fields would thus be comparatively more successful.

In the present work, we used a rice field isolate of *A. siamensis* which has a fast growth rate (0.123 h^{-1}) and high nitrogen-fixing capacity (6). This strain also adapts well to temperature fluctuations (25 to 42°C) prevailing in rice fields and to the salinity ranges (1 to 2%) existing in most of the tropical wetland soils (5). We hereby describe the characterization of an ammonium-excreting mutant of *A. siamensis*.

* Corresponding author.

MATERIALS AND METHODS

Organism. The nitrogen-fixing cyanobacterium *A. siamensis* used in this study, originally isolated in Thailand (6), was obtained from the Sammlung von Algenkulturen, Pflanzenphysiologisches Institute, Universität Göttingen, Göttingen, Federal Republic of Germany, under the signature *Anabaena* sp. strain B 11.82.

Growth conditions. *A. siamensis* was cultivated in AS medium (1) in 500-ml sterilized glass columns placed in a transparent Plexiglas circulating water bath. Water temperature was maintained at 42°C. A constant photon flux of 175 microeinsteins 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 to 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 (7). The cultures were maintained at 5 μg of chlorophyll ml^{-1}.

Immobilization was carried out by using the alginate entrapment method (11) with a few modifications. Cyanobacterial cell suspensions containing 10 μg of chlorophyll ml^{-1} were mixed with an equal volume of 3% sodium alginate solution and added dropwise into 0.1 M $CaCl_2$ solution through a capillary tube to form beads of 2 to 5 mm in diameter. The alginate-entrapped cells were collected after 2 h and kept at 4°C for 12 h. The beads were loaded into packed-bed reactors to collect the ammonium released into the culture medium with a dilution rate of 0.2 h^{-1}.

Growth was followed by means of chlorophyll and protein determinations; the initial inoculum contained 1 μg of chlorophyll ml^{-1}, which corresponded to a protein concentration of 26.3 μg ml^{-1}. Chlorophyll *a* was determined colorimetrically in methanol extracts (17), and protein was determined

by the method of Lowry et al. (16) after digestion with 0.5 N NaOH.

Mutagenesis. Mutagenesis with ethyl methanesulfonate was carried out as reported by Spiller et al. (26). Mutants resistant to MSX were selected on agar plates containing 500 μM MSX. Ammonium-excreting mutants were selected on plates containing the pH indicator phenol red. One of the mutants, designated as SS1, caused a strong change of color on the plates and was used for further studies.

Phycocyanin determinations. Five milliliters of cell suspension was centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was suspended in the same volume of 20 mM sodium acetate buffer, pH 5.5, containing 3 mM sodium azide and 10 mM disodium EDTA. Cells were broken by sonication at 4°C and centrifuged at 10,000 × *g* for 10 min at 4°C to remove membrane fragments containing chlorophyll. Phycocyanin concentration was calculated from measurements of optical densities at 620 and 650 nm (28).

Heterocyst frequencies. Heterocyst frequencies were determined microscopically. Ten independent counts of about 100 vegetative cells each were made for each sample.

Ammonium uptake. Ammonium uptake was followed for 20 min starting from an initial concentration of 100 μM, as described previously (32). Cell concentration for the treatments corresponded to 120 μg of protein ml^{-1}.

Ammonium determinations. Culture filtrates were collected during growth and analyzed for the amounts of ammonium released into the medium by Solorzano's phenol-hypochlorite method (25).

Enzyme assays. Nitrogenase activities were estimated in intact filaments by the acetylene reduction assay (27). Samples of 4.6 ml of culture were washed, suspended in fresh AS medium, and placed in 25-ml Wheaton bottles sealed with a flanged rubber septum. The Wheaton bottles were placed on a rotary shaker (100 rpm) and illuminated with a quantum flux of 75 microeinsteins 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, using a stainless-steel column packed with Porapack-N (0.2-cm inside diameter, 265-cm length). Nitrogenase activities were expressed as micromoles of C_2H_4 produced per milligram of chlorophyll per hour.

Nitrogenase activities in immobilized cyanobacteria were determined as described above by incubating the alginate beads (with 5 μg of chlorophyll ml^{-1}) in 130-ml Wheaton bottles.

Glutamine synthetase (GS) activities were assayed in concentrated suspensions (1 mg of protein ml^{-1}) of cells which were permeabilized with 2% toluene for 1 min and then kept on ice for 15 min. Activity was measured as transferase and expressed in micromoles of γ-glutamyl hydroxamate formed per milligram of protein per minute (23). For in vitro GS activity determinations, 500 μM MSX was added to the enzyme assay mixture.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Exponentially growing cells were harvested and suspended in 20 ml of ice-cold Tris hydrochloride buffer (pH 8.0)–2 mM disodium EDTA–3 mM dithiothreitol–5 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride. The cells were lysed by passage through a French pressure cell (113 kg cm^{-2}) and then centrifuged (10,000 × *g*) for 30 min at 4°C to remove debris and unbroken cells. Soluble polypeptides were precipitated by addition of trichloroacetic acid (to 5%), and the pellet recovered after centrifugation was washed with ice-cold acetone. The proteins were suspended in Laemmli's breaking buffer, boiled for 5 min, separated by electrophoresis through 10% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) sodium dodecyl sulfate (13), and then stained with Coomassie blue G. The molecular weights of standard polypeptides (Sigma Chemical Co.) used were as follows: 200,000 (myosin); 116,250 (β-galoctosidase); 97,400 (phosphorylase *b*); 66,200 (bovine albumin); 45,000 (egg albumin); 31,000 (carbonic anhydrase).

Immunoblot analysis. Following electrophoresis, proteins were transferred electrophoretically to a nitrocellulose membrane filter (0.45 μm; Sigma Chemical Co.) at 110 V for 1 h in an ABN PolyBlot transfer system (American Bionetics) (29). The GS levels in dot blot and Western blot (immunoblot) analyses were detected with the antiserum against purified *Anabaena* sp. strain 7120 GS by the method of Orr and Haselkorn (21) and the reagents of Stratagene Cloning Systems (picoBlue ImmunoDetection Kit) per the kit directions.

Chemicals. MSX was purchased from Sigma, and other chemicals were from E. Merck AG. Sodium alginate was the product of Aldrich Chemical Co. Chemicals used for polyacrylamide gel electrophoresis were acquired from Bio-Rad Laboratories.

RESULTS

Growth characteristics of SS1. SS1 mutant contained chlorophyll (Fig. 1A) and protein (Fig. 1B) at the same level as the parent throughout the growth cycle. At the optimal temperature (42°C), both mutant and wild-type strains grew with a doubling time of 5.6 h, whether the nitrogen source was molecular nitrogen (Fig. 1) or nitrate (data not shown), attaining the stationary phase on day 3. The doubling time was reduced when ammonium or glutamine were used as the sole nitrogen source (about 4 and 4.5 h, respectively). Both strains grew at the same rate when immobilized (Table 1). SS1 exhibited phycocyanin levels (14.0 μg ml^{-1}) significantly higher than in the parent strain (9.6 μg ml^{-1}). Addition of 500 μM MSX to wild-type liquid cultures caused chlorosis (Fig. 1A), followed by filament lysis (Fig. 1B).

Rate of ammonium release. No detectable ammonium was released by the parent strain during active growth (data not shown). The rate of ammonium production by SS1 during the growth cycle in a batch culture (Fig. 2) was the highest at the 12th hour (8.7 μmol mg of chlorophyll^{-1} h^{-1}) and declined during the entry to the stationary phase at day 2; subsequently, the rate was almost nil. In continuous cultures, on the other hand, the rate of ammonium production was constant, 8.7 μmol mg of chlorophyll^{-1} h^{-1}, similar to the rate at the 12th hour of growth in batch cultures (Table 1). A higher rate of ammonium production, 11.6 μmol mg of chlorophyll^{-1} h^{-1}, was obtained in immobilized cells (Table 1).

Ammonium uptake. Both the parent and mutant strains showed the same pattern of ammonium uptake in the absence of MSX (Fig. 3), 32.6 and 30.1 nmol mg of protein^{-1} min^{-1}, respectively. Addition of 200 μM MSX completely inhibited ammonium uptake only in the parent, but did not change the rate of uptake by SS1, 30.6 nmol μg of protein^{-1} min^{-1}, with or without the inhibitor.

Nitrogenase activity. Both strains exhibited identical frequencies of heterocysts (about 19%) and the same pattern of acetylene reducing activity during the growth cycle (Fig. 4). When the cultures became dense, their nitrogenase activities decreased, probably due to shortage of light available to the cells rather than to the phase of growth: the specific activity of the enzyme immediately after diluting the stationary-

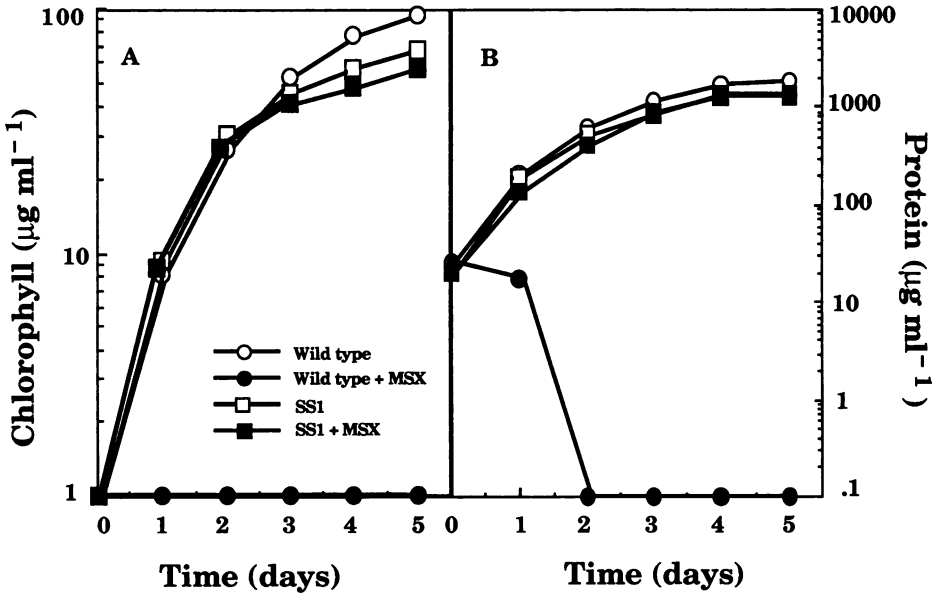

FIG. 1. Growth of the parent *A. siamensis* and its SS1 mutant in the presence and absence of 500 μM MSX, in terms of chlorophyll (A) and protein (B) concentrations.

phase culture was similar to that in log phase (results not shown). Both the parent and mutant strains exhibited acetylene reducing activities in the presence of ammonium, the rate being higher in SS1 (8.9 μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1} versus 1.8 μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1}). Nitrogenase activity was 30% higher in SS1 than in the parent strain at the 12th hour of growth, reaching a value of 35.5 μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1}; in a continuous culture, it was similarly higher (Table 1). The activity in SS1 and not in the parent was further enhanced (16%) by immobilization. Increased nitrogenase activities were observed in other cyanobacterial species following immobilization (8, 19).

GS activity. In the presence of 500 μM MSX, the GS activity of the parent strain was completely inhibited. The mutant strain showed <50% of GS activity in both the presence and the absence of MSX, compared with that of the wild-type strain (without MSX), during the whole growth cycle (Fig. 5) and under immobilizing conditions (Table 1). Similar reduction in SS1 GS activity was also observed during in vitro assay (1.6 μmol of γ-glutamyl hydroxamate mg of protein^{-1} min^{-1} versus 3.1 μmol of γ-glutamyl hydroxamate mg of protein^{-1} min^{-1} in the parent).

Protein analysis. When the total proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gels, the absence of a 30,000-dalton polypeptide was noticed in the profile of SS1 (Fig. 6). Preliminary analysis revealed that it was a soluble cytoplasmic polypeptide (results not shown).

Immunoassay of GS protein. Immunoblot analysis of crude lysates at various dilutions showed similar extents of antigen reaction in the parent and SS1 (Fig. 7A). GS protein levels and its mobility seem to be identical in the parent and SS1 in a Western blot analysis (Fig. 7B).

TABLE 1. Growth rates, enzyme activities, and ammonium excretion rates for the parent and SS1 grown under different growth conditions

Growth conditions[a]	Nitrogenase activity (μmol of C_2H_4 mg of chlorophyll^{-1} h^{-1})	GS activity (μmol of γ-glutamyl hydroxamate mg of protein^{-1} min^{-1})	Rate of NH_4^+ excretion (μmol mg of chlorophyll^{-1} h^{-1})
Batch culture			
Parent	21.0	3.6	0.0
SS1	35.5[b]	1.3	8.7[b]
Continuous culture			
Parent	24.5	3.0	0.0
SS1	35.3	1.4	8.7
Immobilized culture			
Parent	20.5	3.1	0.0
SS1	41.3[b]	1.2	11.6[b]

[a] Doubling time was 5.6 h under all conditions for both parent and SS1.
[b] Measured after 12 h of inoculation.

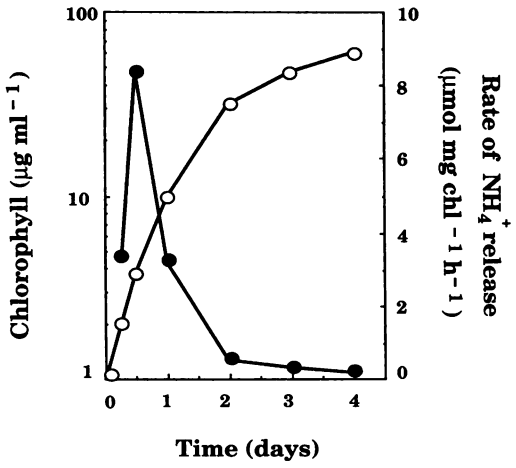

FIG. 2. Chlorophyll content (○) and rate of ammonium release (●) during the growth cycle of the SS1 mutant.

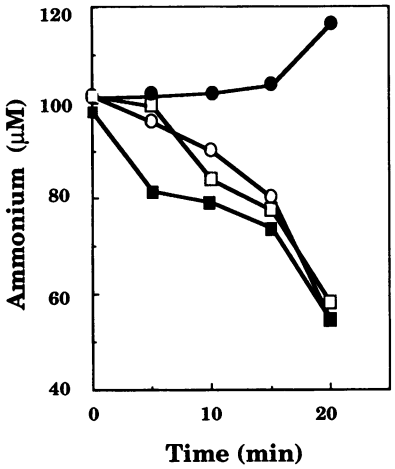

FIG. 3. Ammonium uptake by the parent (○) and SS1 (□). Closed symbols are treatments with MSX.

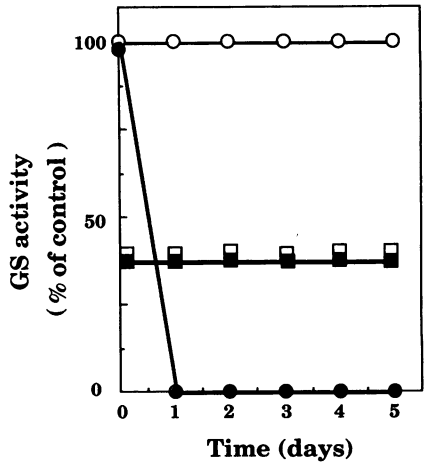

FIG. 5. GS activity in the parent and SS1 strains grown with and without MSX. Closed symbols indicate the addition of MSX. Circles and squares represent the parent and the mutant, respectively.

DISCUSSION

The contribution of nitrogen-fixing cyanobacteria to the productivity of rice fields has long been recognized (30), but the inoculation of nonindigenous cyanobacterial strains is not as successful as expected due to failure to overcome the interspecific competition and environmental constraints. The need thus arises to identify good strains among the native populations of nitrogen-fixing cyanobacteria having high potentials of increasing the yield of rice plants. In this respect, *A. siamensis* proved promising (5) and is already marketed as an algal biofertilizer for rice fields. Its efficiency in increasing the growth and yield of rice plants is apparently due to its high nitrogen-fixing capacity (2, 3). It was reported to release a variety of amino acids during active growth (4), but not ammonium as observed for other nitrogen fixers found in rice fields (30). It does release ammonium into the medium in the presence of MSX, an inhibitor of GS activity (results not shown). Selection of mutants resistant to MSX was found to lead to reduction in GS activity (11). The unassimilated ammonium is consequently released into the medium without induction by MSX (24, 26). It is assumed that a strain releasing ammonium continuously would be a better biofertilizer (14). The MSX-resistant mutant of *A. siamensis* isolated in this study, SS1, seems to conform to this expectation.

SS1 released ammonium due to the high activity of nitrogenase, both being controlled by the cell density of the culture (Fig. 2 and 4). The direct effector was apparently light availability, which became progressively limited as cell density increased. The rate of ammonium release was consequently maximum only during the early log phase of growth in batch cultures. A similar pattern was seen under immobilized conditions.

Based on the above observations, SS1 growing in continuous cultures at low cell densities (chlorophyll value of 5 to 7 $\mu g\ ml^{-1}$) seems an ideal system for sustained ammonium release. The rate of ammonium release by SS1 was lower than the rates obtained for other mutants of *A. variabilis*, i.e., 35 to 50 μmol mg of $chlorophyll^{-1}\ h^{-1}$ in batch and immobilized cultures (11, 26). No data have been provided, however, on the excretion of ammonium by these mutants under steady-state growth conditions. Hence, comparison of rates of ammonium release by the various mutants is inade-

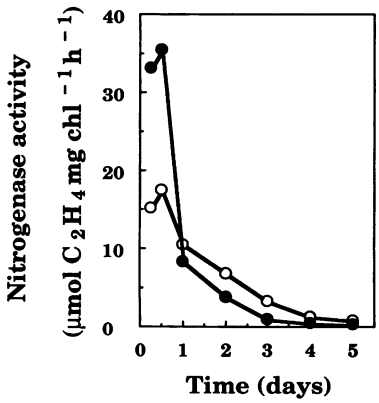

FIG. 4. Nitrogenase activity of the parent (○) and SS1 (●) during growth in batch cultures expressed by acetylene reduction.

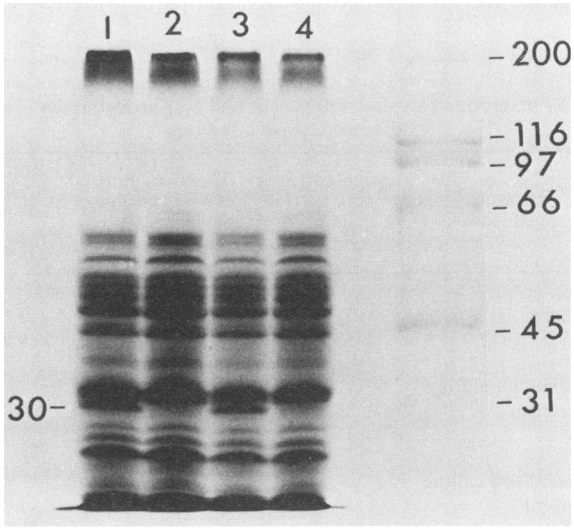

FIG. 6. Separation of total proteins from parent (lanes 1 and 3) and SS1 grown in the absence (lane 2) and presence (lane 4) of 500 μM MSX.

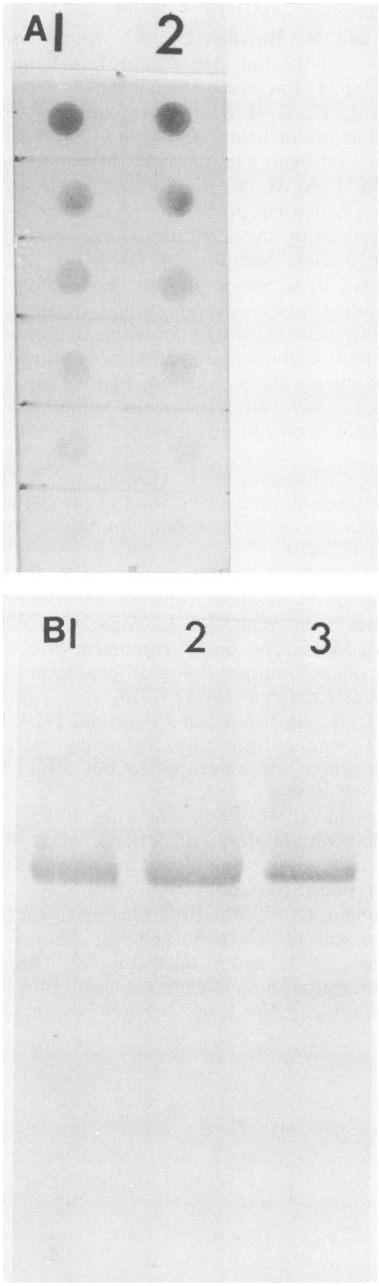

FIG. 7. Immunoblot analysis (A) of total proteins in the parent strain (1) and the mutant SS1 (2). Crude lysates were dotted from top to bottom with the following amounts of protein: 100, 50, 25, 12.5, 6.25, and 3.125 ng. Western blot analysis (B) of GS protein: lane 1, SS1 grown in the absence of MSX; lane 2, SS1 grown in the presence of MSX; lane 3, parent.

quate. The phycocyanin content of SS1 was also higher than of the parent, and it is possible that a certain proportion of the fixed nitrogen is expended for the synthesis of this storage product (7).

Nitrogenase activity in SS1 was 30 and 50% higher than that of the parent during steady-state growth (Fig. 4) and under immobilizing conditions (Table 1), respectively. In the presence of ammonium, SS1 nitrogenase activity was about fivefold higher than that in the parent due to a weaker repression by the end product (22). GS seemed to be defective in SS1 as well, exhibiting <50% of the parent enzyme activity (Fig. 5) without a significant reduction in protein content (Fig. 7A). It therefore appears that the decreased susceptibility of nitrogenase activity to repression by ammonium and the defective activity of GS are related to ammonium excretion in SS1. The ammonium-excreting mutants of *A. variabilis* (9), i.e., SA1, ED81, and ED92, were found to have derepressed nitrogenase and lower GS activities. Analyses of GS and its mRNA in the two ethylenediamine-resistant mutants (9) suggested that one of them (ED92) was a regulatory mutant containing less GS mRNA and consequently less GS protein as found for *A. azollae* growing in symbiosis (20). The other (ED81) is a structural mutant with a catalytically deficient GS, resulting in reduced activities, which synthesizes protein in equal amounts to its parent (12), as found for *Nostoc* sp. strain 7801 growing in symbiosis (10, 15). Immunoblot analysis of crude proteins and Western blot analyses of GS from SS1 revealed that its quantity (Fig. 7A) and mobility (Fig. 7B) are the same as in the parent. A modification similar to that of ED81 may also have occurred in SS1, forming a catalytically deficient GS, but conclusive evidence is yet to be obtained.

A 30,000-dalton soluble cytoplasmic protein was absent from SS1 grown in the presence and absence of MSX (Fig. 6). Its relevance to nitrogen metabolism should be elucidated.

Fertilization of rice plants under laboratory conditions by application of another MSX-resistant mutant of *A. variabilis*, SA1, was successful (14). The shorter doubling time of SS1 and the lack of a lag period at the beginning of the growth cycle as compared with SA1 (26) are obvious assets for SS1 mass cultivation. These characteristics enable efficient production of inoculum material. The usefulness of SS1 as a biofertilizer to rice plants in the original isolate location (Thailand) should be studied further.

ACKNOWLEDGMENTS

This work was supported by AID-CDR grant DPE-5544-G-SS-6036-00.

We thank Robert Haselkorn for providing the GS antibodies, Aharon Abeliovich for help with immunoassays, and Dorot Imber for editing the manuscript.

LITERATURE CITED

1. **Antarikanonda, P.** 1982. Effect of salinity on growth, nitrogen fixation and sodium uptake of rapidly growing N_2-fixing blue-green alga *Anabaena* sp. TA1. Microbios **34**:177–184.
2. **Antarikanonda, P.** 1982. Influence of pretreating the seeds with extract of *Anabaena siamensis* on germination and growth of some rice varieties from Thailand. FAO Int. Comm. **31**:37–39.
3. **Antarikanonda, P.** 1982. Influence of nitrogen fixing blue green alga *Anabaena siamensis* on yield and protein quality of highly yielding rice cultivar (Khao Dowk Mali 4-2-105). German Research Society Report no. 10680, p. 30.
4. **Antarikanonda, P.** 1984. Production of extracellular free amino acids by cyanobacterium *Anabaena siamensis*. Curr. Microbiol. **11**:191–196.
5. **Antarikanonda, P.** 1985. A new species of the genus *Anabaena*: *Anabaena siamensis* sp. nov. (Cyanophyceae) from Thailand. Nova Hedwigia **41**:343–352.
6. **Antarikanonda, P., and H. Lorenzen.** 1982. N_2 fixing blue green algae (Cyanobacteria) of high efficiency from paddy soils of Bangkok, Thailand: characterization of species and nitrogen fixing capacity in the laboratory. Acta Hydrobiol. Suppl. **63**:53–70.
7. **Boussiba, S., and A. E. Richmond.** 1980. C-phycocyanin as a storage protein in the blue-green alga *Spirulina platensis*. Arch. Microbiol. **125**:143–147.

8. **Brouers, M., and D. O. Hall.** 1986. Ammonia and hydrogen production by immobilized cyanobacteria. J. Biotechnol. **3**:307–321.
9. **Hien, N. T., N. W. Kerby, G. C. Machray, P. Rowell, and W. D. P. Stewart.** 1988. Expression of glutamine synthetase in mutant strains of the cyanobacterium *Anabaena variabilis* which liberate ammonia. FEMS Microbiol. Lett. **56**:337–342.
10. **Joseph, C. M., and J. C. Meeks.** 1987. Regulation of expression of glutamine synthetase in a symbiotic *Nostoc* strain associated with *Anthoceros punctatus*. J. Bacteriol. **169**:2471–2475.
11. **Kerby, N. W., S. C. Musgrave, P. Rowell, S. V. Shestakov, and W. D. P. Stewart.** 1986. Photoproduction of ammonium by immobilized mutant stains of *Anabaena variabilis*. Appl. Microbiol. Biotechnol. **24**:42–46.
12. **Kerby, N. W., G. W. Niven, P. Rowell, and W. D. P. Stewart.** 1987. Ammonia and amino acid production by cyanobacteria, p. 277–286. *In* T. Stadler, J. Mollion, M.-C. Verdus, Y. Karamanos, H. Morvan, and D. Christiaen (ed.), Algal biotechnology. Elsevier Applied Science, London.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lattore, C., J. H. Lee, H. Spiller, and K. T. Shanmugam.** 1986. Ammonium ion-excreting cyanobacterial mutant as a source of nitrogen for growth of rice: a feasibility study. Biotechnol. Lett. **8**:507–512.
15. **Lee, K. Y., C. M. Joseph, and J. C. Meeks.** 1988. Glutamine synthetase specific activity and protein concentration in symbiotic *Anabaena* associated with *Azolla caroliniana*. Antonie van Leeuwenhoek J. Microbiol. Serol. **54**:345–355.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Mackinney, G.** 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. **140**:315–322.
18. **Martinez, M. R.** 1984. Algae: biofertilizer for rice. Philipp. Counc. Agric. Res. Resources Dev. (PCARRD) Monit. **12**:9–12.
19. **Musgrave, S. C., N. W. Kerby, G. A. Codd, and W. D. P. Stewart.** 1982. Sustained ammonia production by immobilized filaments of the nitrogen fixing cyanobacterium *Anabaena* 27893. Biotechnol. Lett. **4**:647–652.
20. **Nierzwicki-Bauer, S. A., and R. Haselkorn.** 1986. Differences in mRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*. EMBO J. **5**:29–35.
21. **Orr, J., and R. Haselkorn.** 1982. Regulation of glutamine synthetase activity and synthesis in free living and symbiotic *Anabaena* sp. J. Bacteriol. **152**:626–635.
22. **Polukhina, L. E., G. N. Sakhurieva, and S. V. Shestakov.** 1982. Ethylenediamine-resistant *Anabaena variabilis* mutants with derepressed nitrogen fixing system. Microbiology **51**:90–95.
23. **Sampio, M. J. A. M., P. Rowell, and W. D. P. Stewart.** 1979. Purification and some properties of glutamine synthetase from the nitrogen fixing cyanobacteria *Anabaena cylindrica* and a *Nostoc* sp. J. Gen. Microbiol. **111**:181–191.
24. **Singh, H. N., R. K. Singh, and R. Sharma.** 1983. An L-methionine-D,L-sulfoximine-resistant mutant of the cyanobacterium *Nostoc muscorum* showing inhibitor resistant γ-glutamyl-transferase, defective glutamine synthetase and producing extracellular ammonia during N_2 fixation. FEBS Lett. **153**:10–14.
25. **Solorzano, L.** 1969. Determination of ammonia in natural waters by the phenol-hypochlorite method. Limnol. Oceanogr. **14**:799–801.
26. **Spiller, H., C. Latorre, M. E. Hassan, and K. T. Shanmugam.** 1986. Isolation and characterization of nitrogenase derepressed mutant strains of cyanobacterium *Anabaena variabilis*. J. Bacteriol. **165**:412–419.
27. **Stewart, W. D. P., G. P. Fitgerald, and R. H. Burris.** 1967. *In situ* studies on N_2 fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. USA **58**:2071–2078.
28. **Tandeau de Marsac, N., and J. Houmard.** 1988. Complementary chromatic adaptation: physiological conditions and action spectra. Methods Enzymol. **167**:318–328.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
30. **Venkataraman, G. S.** 1975. The role of blue green algae in tropical rice cultivation, p. 207–218. *In* W. D. P. Stewart (ed.), Nitrogen fixation by free-living microorganisms. Cambridge University Press, London.
31. **Venkataraman, G. S.** 1981. Blue-green algae: a possible remedy to nitrogen scarcity. Curr. Sci. **50**:253–256.
32. **Zimmerman, W. J., and S. Boussiba.** 1987. Ammonia assimilation and excretion in an asymbiotic strain of *Anabaena azollae* from *Azolla filiculoides* Lam. J. Plant Physiol. **127**:443–450.