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Isolation and Characterization of Nitrogenase-Derepressed Mutant Strains of Cyanobacterium *Anabaena variabilis*†

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A positive selection method for isolation of nitrogenase-derepressed mutant strains of a filamentous cyanobacterium, *Anabaena variabilis*, is described. Mutant strains that are resistant to a glutamate analog, L-methionine-D,L-sulfoximine, were screened for their ability to produce and excrete NH_4^+ into medium. Mutant strains capable of producing nitrogenase in the presence of NH_4^+ were selected from a population of NH_4^+-excreting mutants. One of the mutant strains (SA-1) studied in detail was found to be a conditional glutamine auxotroph requiring glutamine for growth in media containing N_2, NO_3^-, or low concentrations of NH_4^+ (less than 0.5 mM). This glutamine requirement is a consequence of a block in the assimilation of NH_4^+ produced by an enzyme system like nitrogenase. Glutamate and aspartate failed to substitute for glutamine because of a defect in the transport and utilization of these amino acids. Strain SA-1 assimilated NH_4^+ when the concentration in the medium reached about 0.5 mM, and under these conditions the growth rate was similar to that of the parent. Mutant strain SA-1 produced L-methionine-D,L-sulfoximine-resistant glutamine synthetase activity. Kinetic properties of the enzyme from the parent and mutant were similar. Mutant strain SA-1 can potentially serve as a source of fertilizer nitrogen to support growth of crop plants, since the NH_4^+ produced by nitrogenase, utilizing sunlight and water as sources of energy and reductant, respectively, is excreted into the environment.

In free-living, nitrogen-fixing organisms, nitrogenase synthesis and activity are regulated by the presence of NH_4^+ in the medium (3, 10, 31, 45). That NH_4^+-mediated regulation of nitrogenase synthesis is not exerted by NH_4^+ itself but is actually a consequence of metabolic products of NH_4^+ assimilation has been revealed by several lines of evidence. (i) Mutant strains incapable of NH_4^+ assimilation were derepressed for nitrogenase synthesis in the presence of NH_4^+ (28, 31, 43, 44). (ii) Addition of amino acids to the growth medium repressed nitrogenase synthesis even in nitrogenase-derepressed mutant strains (30). (iii) Inhibition of glutamine synthetase, a primary enzyme responsible for NH_4^+ assimilation, by a substrate (glutamate) analog, L-methionine-D,L-sulfoximine (MSX) as well as by other inhibitors led to derepression of nitrogenase synthesis in the presence of NH_4^+ in all organisms tested (8, 13, 14, 21, 31, 39). The above described derepression of nitrogenase synthesis is achieved by alteration of cellular physiology leading to a decrease in the rate of glutamate production, since addition of amino acids to the medium reversed the effect (22, 29). This set of conditions also derepressed other NH_4^+- as well as glutamate-producing enzyme systems (NO_3^- assimilation; histidine and proline utilization) in the cell (16, 29).

Mutant strains that are blocked at the level of NH_4^+ assimilation were first described in *Klebsiella pneumoniae* (32), and such mutant strains not only derepress nitrogenase synthesis but also excrete the NH_4^+ produced by the enzyme into the medium (32, 44). Since that time, nitrogenase-derepressed mutants have been isolated from several different organisms, including cyanobacteria (7, 27, 40, 43, 44). Heterotrophic, nitrogen-fixing organisms utilize organic compounds as sources of energy and reductant for nitrogen fixation, and the high energy demand of the nitrogen-fixing cell (2) is met by heterotrophic metabolism. On the other hand, cyanobacteria use sunlight and water as sources of energy and reductant for nitrogen fixation (10, 45). Because of this physiological property, nitrogenase-derepressed mutant strains of the ubiquitous, heterocystous cyanobacteria can potentially be used as suppliers of fertilizer nitrogen for plant growth.

We have recently described the isolation and partial characterization of a nitrogenase-derepressed mutant strain of *Anabaena variabilis* (37). Using a modification of the procedure used to isolate this mutant of *A. variabilis*, we isolated several nitrogenase-derepressed mutant strains, and the physiological and biochemical properties of these mutants are presented in this report.

MATERIALS AND METHODS

Materials. Biochemicals were purchased from Sigma Chemical Co. Inorganic and organic chemicals were obtained from Fisher Scientific Co. and were of analytical grade.

Bacterial strains. All cyanobacterial mutants used in this study were derivatives of wild-type *A. variabilis* Kütz (ATCC 29413), described as strain SA-0 in this study.

Media and growth conditions. A chemically defined medium (A medium) described by Allen and Arnon (1) was used at one-half strength (A/2). This medium was supplemented with fructose (5 mM) to enhance the growth rate of the culture. Phosphate (3 mM) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (10 mM) served as buffers (11). The pH of the final medium was 7.0. This medium, defined as BGA medium, was supplemented with different nitrogen sources, as indicated for different experiments. BGA medium was supplemented with L-glutamine (2 mM)–L-glutamate (0.5 mM)–L-aspartate (1 mM)–L-methionine (1 mM)–MSX (0.5 mM) for selection of mutant strains that were resistant to this glutamate analog, and this medium is called MSX medium. Various amino acids were included in the

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medium to support the growth of any auxotrophic mutant strain that might arise in the MSX medium during selection. Methionine was included in this medium, since bacteria are known to transport MSX by both the glutamate-glutamine and methionine transport systems (4, 6, 12). The ability to alter the pH of the culture medium was determined by using pH indicator dyes with a narrow pH range in a weakly buffered medium. Two different pH indicators, bromothymol blue (0.002%) and phenol red (0.0025%), were found to be suitable. The pH indicator medium consisted of A/2 medium containing fructose (5 mM), aspartate (1 mM), and glutamine (1 mM). Phosphate at a final concentration of 0.2 mM served as the only buffer in the medium. The final pH of this medium was 6.7. An increase in pH of 0.2 altered the color of the medium from green to blue for bromothymol blue and from orange to red for phenol red. Solid medium contained 15 g of agar per liter.

Cultures were grown with continuous illumination (5,000 lx) in a shaker at 30°C. Growth of the culture was monitored by the increase in optical density at 750 nm (OD_{750}) (Bausch and Lomb, Inc.; Spectronic 710 spectrophotometer). Before determining the OD of the culture, the samples were sonicated in a Sonicator bath (Branson Cleaning Equipment Co. ultrasonic cleaner, model B-12) for 15 s, to reduce filament length. This step provided a uniform cell suspension and reproducibility of the OD measurements (35). An OD_{750} value of 2.4 corresponds to 1 mg (dry weight) of cells per ml under our experimental conditions.

Mutagenesis. The procedure used for ethyl methanesulfonate (EMS) mutagenesis of *A. variabilis* was adapted from the one described for *K. pneumoniae* by Shanmugam and Valentine (33). Cells from a 50-ml culture at mid-exponential phase of growth in BGA medium with N_2 as the nitrogen source were harvested by centrifugation at 3,500 rpm at room temperature. The filaments were washed once in A/2 medium and suspended in 12 ml of BGA medium containing NH_4^+ (5 mM) as the nitrogen source. This cell suspension (5×10^8 CFU/ml) was sonicated in a sonicator bath in 2.5-ml portions for 10 to 15 s to reduce filament length to about 5 to 8 cells per filament. Survival rate for this step was about 85%, based on microscopic determination and CFU/ml. Shorter filament length reduced the survival rate substantially. The cell suspension was washed once after sonication and suspended in the same medium. EMS was added to this culture at a final concentration of 1.0%. The culture was incubated in a shaker at room temperature for 90 min with continuous illumination. The mutagenized cells were washed once with A/2 medium and suspended in A/2 medium. Treatment with the mutagen reduced the surviving fraction to 22%. The filaments obtained after mutagenesis were incubated at 48°C for 40 min to aid in depurination of alkylated guanine residues, and this step reduced the surviving population to about 5% of the zero time value. The cells were collected by centrifugation and suspended in BGA medium with NH_4^+ (3 mM) at a final cell density of 6×10^7/ml. This culture was incubated in light at room temperature with gentle shaking (100 rpm) for 10 h to allow for segregation of the mutagenized chromosome. A control culture was carried through the same procedure without EMS treatment, and the survival level for this control was about 39% after these various steps. As a measure of the effectiveness of the mutation procedure, EMS treatment increased the frequency of streptomycin resistance by about 30 times over spontaneous levels and that of resistance to 5-fluorocytosine by about 40 times.

Enzyme assays. Nitrogenase activity was determined with whole cells as previously described (35). Glutamine synthetase activity was determined with crude extracts from cultures at the mid- to late-exponential phase of growth as described by Orr and Haselkorn (20). Crude extract for glutamine synthetase assay was prepared as described earlier (28). Nitrate and nitrite reductase activities were determined with whole cells. Cells were washed and suspended in phosphate buffer (50 mM; pH 7.0) containing kanamycin sulfate (70 μg/ml) to prevent protein synthesis during the assay. For determination of nitrate reductase activity, KNO_3 (20 mM) was added to the cells (in the assay medium) and incubated in light. Samples were removed at different time intervals, and the amount of NO_2^- produced was determined after removing the cells by centrifugation. Nitrite reductase activity was determined as described by Spiller et al. (36) with an assay mixture containing Tricine (Sigma) buffer (50 mM; pH 8.0), $NaNO_2$ (1 mM), methyl viologen (1 mM), and dithionite (10 mM). Protein was determined by previously published procedures (5, 15). Bovine serum albumin served as the standard.

NH_4^+ determination. Spent medium from an appropriate culture was obtained after removing the cells by centrifugation at room temperature. Concentration of NH_4^+ in the spent medium was determined after Conway diffusion with Nessler reagent (2).

Amino acid incorporation. The incorporation of amino acids into cell material was followed with ^{14}C-labeled amino acids (ICN Biomedicals Inc.) at a specific activity of 15 μCi/mmol. The amino acid concentration in the A/2 medium supplemented with fructose was 3 mM at the beginning of the growth experiment. At different time intervals, samples were removed from the culture to monitor growth and incorporation of the label into cell material. Cells were collected on a glass fiber filter (GF/C; Whatman, Inc.) and washed with cold A/2 medium followed by trichloroacetic acid (5%) and ethanol (95%). The amount of radioactivity in the cells (filter) was determined with a scintillation counter after adding 3 ml of Bray solution.

RESULTS

Isolation of mutants. Although mutant strains capable of producing nitrogenase activity in the presence of NH_4^+ have been described in other organisms, general procedures for the isolation of similar mutant strains of cyanobacteria have not been reported. We have devised a selection procedure for isolation of nitrogenase-derepressed mutants of cyanobacteria, which is based on the properties of similar mutants of *K. pneumoniae* (28). The results obtained with *K. pneumoniae* mutants indicated that a genetic block at the level of NH_4^+ assimilation leads to production of nitrogenase in the presence of NH_4^+. These mutant strains also excreted the NH_4^+ produced by nitrogenase into the medium. (31, 32).

A. variabilis is known to assimilate NH_4^+ by using the enzymes glutamine synthetase and glutamate synthase (17). In cyanobacteria and all other organisms studied so far, inhibition of glutamine synthetase activity by glutamate analogs leads to production of nitrogenase in the presence of NH_4^+ (14, 17, 18, 26, 31). Based on the above observations, a mutation affecting the production of either of the two NH_4^+ assimilation enzymes in an active state should lead to derepression of nitrogenase synthesis in the presence of NH_4^+. However, such mutants would be either glutamine or glutamate auxotrophs, depending on the location of the genetic lesion, and would require the appropriate amino acid for growth. An alternate method would be the genetic alteration of the rate of NH_4^+ assimilation in such a way that

the mutant strain cannot assimilate NH_4^+ when it is present at lower concentrations, as is the case with enzyme-catalyzed NH_4^+ production (nitrogenase, nitrate assimilation, etc.), but can utilize NH_4^+ when present at high concentrations (>1 mM).

Mutant strains containing enzymes with altered kinetic properties can be isolated among a population of substrate analog-resistant mutants (42). Hence, MSX-resistant mutants of *A. variabilis* should also include strains that contain glutamine synthetase with altered kinetic properties. Such mutants would be prototrophic as well as derepressed for nitrogenase biosynthesis, and these can be identified in a population of MSX-resistant mutants by screening in an appropriate medium containing pH indicators, since the production and excretion of NH_4^+ by these mutants leads to an increase in the pH of the culture.

For the isolation of nitrogenase-derepressed mutant strains, cultures of *A. variabilis* mutagenized with EMS were plated (2×10^7 CFU per plate) on MSX-agar medium. The plates were incubated at 30°C with continuous illumination. Mutant strains resistant to MSX were selected from these plates and checked for NH_4^+ production and excretion with pH indicator media. Mutants were streaked on the surface of the medium as patches and incubated under low light intensity (2,000 lx) at 30°C. Those strains that altered the pH of the medium to the alkaline side were selected and used for further studies.

Nitrogenase activity. Since the selection procedure was designed for the isolation of nitrogenase-derepressed mutants, the mutant strains were tested for nitrogenase activity in the absence and presence of NH_4^+ in the medium (Table 1). All of the mutant strains produced nitrogenase activity in medium containing L-glutamine alone (2 mM), although at different levels. Parent strain and mutant strains SA-2, SA-3, and SA-5 produced no detectable nitrogenase activity when grown in an NH_4^+-containing medium. Mutant strains SA-1, SA-4, SA-6, and SA-7 produced nitrogenase in the presence of NH_4^+. Strain SA-8 produced nitrogenase at low but detectable levels in the presence of NH_4^+. These results show that nitrogenase-derepressed mutants can be isolated by the procedure outlined above. However, not all the mutants isolated were derepressed for nitrogenase synthesis in the presence of NH_4^+.

TABLE 1. Levels of nitrogenase activity in the parent and mutant strains

Strain	Nitrogenase activity[a]		
	$-NH_4^+$ (control)	$+NH_4^+$ (2 mM)	+Gln and NH_4^+ (added during assay)
Parent	44	0	0
SA-1	63	45	7
SA-2	16	0	0
SA-3	39	0	0
SA-4	105	34	22
SA-5	23	0	0
SA-6	43	30	18
SA-7	27	10	6
SA-8	70	1	0

[a] Nanomoles · h^{-1} · OD_{750}^{-1}. Control cultures were grown in BGA medium with glutamine (2 mM). To determine the level of nitrogenase derepression, we removed two samples from the NH_4^+ culture and determined the nitrogenase activity of the growing culture with one of the samples. Another sample was diluted three times with fresh medium, and additional glutamine and NH_4^+ (1.5 mM each) were added before determination of nitrogenase activity of the culture because of the low levels of NH_4^+ used in the growth medium.

TABLE 2. Production and excretion of NH_4^+ by the parent and mutant strains

Strain	NH_4^+[a]	Cell yield[b] (OD_{750})
Parent	0	2.4
SA-1	1.08	2.1
SA-2	0.25	1.8
SA-3	0.15	2.1
SA-4	0.22	2.3
SA-5	1.42	0.5
SA-6	1.45	0.7
SA-7	0.17	2.3
SA-8	0.54	1.0

[a] Micromoles of NH_4^+ · ml of culture medium^{-1} after 4 days of growth.
[b] Cell yield after 4 days of photoheterotrophic growth in BGA medium.

Production and export of NH_4^+. All the mutants tested did produce NH_4^+ from N_2 and excrete the NH_4^+ into the medium (Table 2). The culture supernatant obtained from strains SA-1, SA-5, and SA-6 had higher levels of NH_4^+ (greater than 1 mM) compared with the spent media from other strains. The parent strain coupled the production and assimilation of NH_4^+ to cell growth, and free NH_4^+ was not a normal constituent of the culture medium under N_2-dependent growth. All the mutant strains that excreted NH_4^+ at a reduced rate (<0.25 mM) did produce high cell yields. Strain SA-8 produced NH_4^+ at an intermediate level (0.54 mM), and the cell yield was also moderate. Strains SA-5 and SA-6 produced lower cell yields under nitrogen-fixing growth conditions, probably because they excreted much of the fixed N. Strain SA-1 is an exception to this general rule and is described in detail below.

Growth characteristics. The lower cell yields observed with the mutant strains that excrete NH_4^+ at high rates (strains SA-5, SA-6, and SA-8; Table 2) indicate that these strains are defective in the assimilation of NH_4^+ produced by nitrogenase. The assimilation of NO_3^- as an N source requires the enzymatic conversion of NO_3^- to NH_4^+, and the regulation of NO_3^- assimilation is similar to nitrogen fixation (23, 29). The results presented in Table 3 show that strains SA-5 and SA-6 grew at a lower rate in media containing N_2 or NO_3^- as the sole N source. However, these two strains failed to grow in NH_4^+-containing medium. Strain SA-1 grew well in the presence of NH_4^+. Strain SA-1 did grow in N_2 as well as in NO_3^- media after a long lag period (see below). Strains SA-1, SA-5, and SA-6 grew in glutamine-supplemented growth medium at about the same growth rate as the parent strain. Strain SA-4 and the parent strain grew in all media tested.

Nitrate assimilation. Since the mutant strains exhibited different levels of derepression for nitrogen fixation, it was of

TABLE 3. Growth characteristics of selected MSX-resistant mutant strains in the presence of different nitrogen sources

Strain	Growth rate[a]			
	Glutamine	NH_4^+	NO_3^-	N_2
Parent	18	11	12	10
SA-1	10	12	43	39[b]
SA-4	11	6	16	16
SA-5	11	NG[c]	29	48
SA-6	7	NG	29	23

[a] Growth rate is expressed as generation time in hours.
[b] See the text for details.
[c] NG, No detectable growth.

TABLE 4. Nitrate reductase and nitrite reductase activities of MSX-resistant mutant strains of *A. variabilis* grown in the presence of different nitrogen sources[a]

Strain	Nitrate reductase			Nitrite reductase		
	$+NO_3^-$	$+NH_4^+$	$+NH_4^+ + NO_3^-$	$+NO_3^-$	$+NH_4^+$	$+NH_4^+ + NO_3^-$
Parent	3.6	0.0	2.7	9.5	0.0	8.0
SA-1	0.3	0.2	0.3	6.0	5.0	5.0
SA-3	5.0	1.0	4.0	17.0	12.0	21.0
SA-4	6.0	0.0	3.0	8.0	0.0	18.0
SA-5	16.0	2.0	4.0	22.0	4.0	16.0
SA-6	16.0	2.0	2.0	40.0	24.0	24.0
SA-7	4.0	1.0	4.0	24.0	0.0	22.0
SA-8	0.0	0.0	0.0	0.0	0.0	0.0

[a] Activity is expressed as nanomoles · h^{-1} · OD_{750}^{-1}. Growth medium for all the cultures contained L-glutamine (2 mM). Concentrations of NO_3^- and NH_4^+ in the medium were 15 and 3 mM, respectively. Cells were harvested and assayed for the two enzyme activities at early- to mid-exponential phase of growth when the medium concentration of NH_4^+ in NH_4^+-containing cultures exceeded 1 mM.

interest to determine whether the derepression extended to enzymes in the nitrate assimilation pathway, another enzyme system involved in the production of NH_4^+. For this purpose, wild-type and mutant strains were grown in different media, and the levels of nitrate reductase and nitrite reductase activities were determined. The parent strain produced nitrate reductase and nitrite reductase activities only in medium containing nitrate (Table 4). In medium lacking nitrate, the two activities were not detectable. The presence of ammonium ion and glutamine in the growth medium had very little effect on the levels of nitrate and nitrite reductases in the parent strain. Similar results were also obtained with strain SA-4. On the other hand, strains SA-1, SA-3, SA-5, and SA-6 produced both enzyme activities even when cultured in medium lacking nitrate. The actual levels of the two enzyme activities varied among the different strains, with strain SA-1 producing very low but detectable levels of nitrate reductase. Strain SA-7 produced low levels of nitrate reductase activity in the absence of NO_3^-. Nitrite reductase activity was not detectable in these cells. However, during the late-exponential phase of growth, strain SA-7 produced both enzyme activities, a probable consequence of NH_4^+ depletion from the medium. Nitrate and nitrite reductase activities were not detected in strain SA-8. These results indicate that some of the MSX-resistant mutants are capable of producing the enzymes responsible for nitrate assimilation in a constitutive manner.

Levels of glutamine synthetase. Although mutant strains SA-5 and SA-6 did not grow in NH_4^+-containing medium, none of the mutant strains tested was a glutamine auxotroph (Table 5). Glutamine synthetase activity could be detected in the crude extracts of all the mutant strains tested, although the levels varied considerably among the strains, from about 5% (strain SA-1) to greater than 100% (strain SA-4) of the observed parental values. Under the experimental conditions used, the ratio of glutamine synthetase biosynthetic activity to γ-glutamyltransferase activity for the parent was 0.25. For most of the mutant strains, this value was similar (0.15 to 0.22) to that of the parent. Two mutant strains, SA-1 and SA-5, produced glutamine synthetase with a lower ratio of biosynthetic to transferase activity (0.07 and 0.11, respectively), suggesting that the biosynthetic activity of glutamine synthetase was adversely affected in these two strains. This decrease in the ratio of the two catalytic activities suggests that the mutation leading to resistance to MSX altered the catalytic properties of glutamine synthetase in the cell.

Growth and NH_4^+ production by strain SA-1. Among the strains tested, SA-1 exhibited unique and interesting characteristics. This strain produced nitrogenase even in the presence of NH_4^+ and excreted the NH_4^+ produced by nitrogenase into the growth medium. Although this strain grew at the expense of N_2 after a long lag period, it produced higher cell yields and was capable of utilizing NH_4^+ as an N source. For these reasons, strains SA-1 was investigated further.

Strain SA-1 was cultured under photoautotrophic and photoheterotrophic growth conditions with N_2 as the sole N source (Fig. 1). Under both growth conditions, the parent strain started to grow immediately after transfer to fresh medium. However, strain SA-1 exhibited a lag of about 2 days before growth started. Although the culture did not grow during this time, the cells did catalyze the production of NH_4^+ from N_2, and this NH_4^+ was detected in the growth medium. The mutant culture started to grow after 2 days when the NH_4^+ concentration in the medium reached approximately 0.5 mM. The initial growth rate was similar to the parent value. The final cell yield of the mutant culture under photoheterotrophic conditions (Fig. 1A) was comparable to that of the parent strain. The net NH_4^+ concentration in the medium reached a value as high as 2 mM and then started to decline. Ammonium ion was not detectable in the growth medium of the parent strain SA-0. The mutant strain maintained under an argon atmosphere in N-free medium failed to produce any detectable NH_4^+, indicating the absence of photorespiratory NH_4^+ release from endogenous nitrogen compounds. These results demonstrate that the mutant strain grew at the expense of N_2 as the sole N source, but only after the NH_4^+ concentration reached values near 0.5 mM in the medium.

Mutant strain SA-1 produced nitrogenase in the presence of NH_4^+, but the specific activity declined with increasing NH_4^+ concentration. In glutamine (2 mM)-containing medium, nitrogenase activity was not detectable during early stages of growth. However, nitrogenase activity was observed during the late-exponential phase of growth, a probable result of glutamine depletion from the medium. Since nitrogenase activity is localized in the heterocysts in cyanobacteria, the frequencies of heterocysts in the parent strain and in the mutant strain SA-1 were compared. The wild-type strain had a heterocyst frequency of about 6%, whereas the mutant had a frequency of about 11%. The mutant strain had a higher frequency of heterocysts that were adjacent to each other with two, three, and, at rare occasions, even four in a row. Two adjacent heterocysts that

TABLE 5. Glutamine synthetase activities in the parent and mutant strains

Strain	Glutamine synthetase activity[a]		
	Transferase	Biosynthetic	Biosynthetic/Transferase
Parent	1.71	0.43	0.25
SA-1	0.084	0.006	0.07
SA-2	1.46	0.19	0.13
SA-3	1.08	0.20	0.19
SA-4	2.80	0.40	0.14
SA-5	2.00	0.21	0.11
SA-6	1.50	0.33	0.22
SA-7	1.33	0.20	0.15

[a] Micromoles · min^{-1} · mg of $protein^{-1}$.

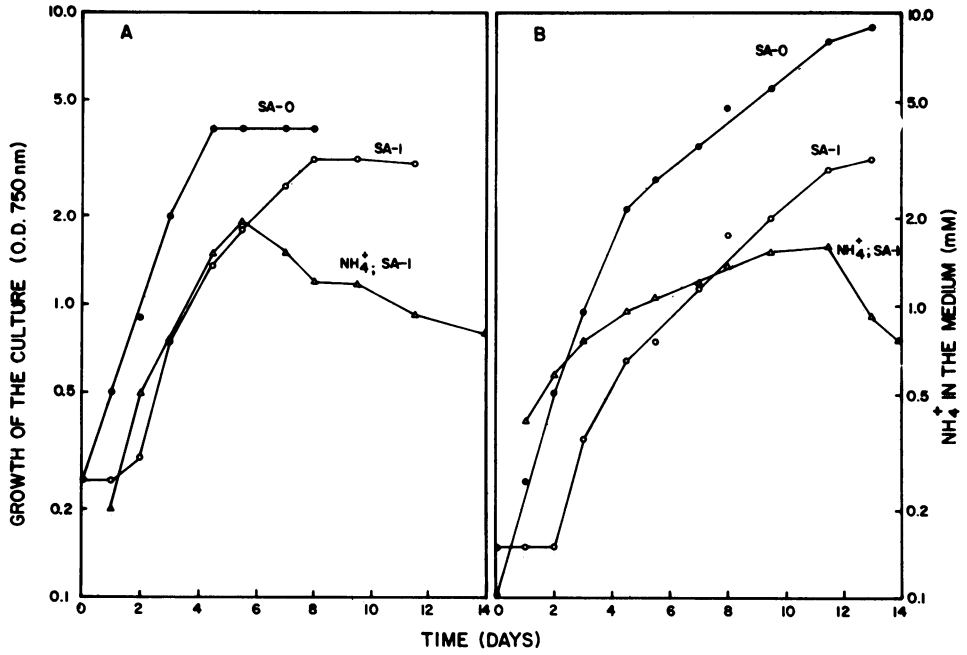

FIG. 1. Growth and NH_4^+ production by wild-type *A. variabilis* (strain SA-0) and MSX-resistant mutant strain SA-1. (A) Photoheterotrophic growth; (B) continuous sparging with air-CO_2 (0.8%) at a rate of 30 ml/min.

were distinct from akinetes could occasionally be observed in the parent strain but occurred about eight times more frequently in the mutant. About 60% of the heterocyst locations in the mutant strain had multiple heterocysts. On the other hand, continuous growth of the mutant strain in the presence of NH_4^+ and glutamine (3 mM each) did lead to a decline in heterocyst frequency to about 5%, but heterocysts were never depleted from the filaments. These results show that both heterocyst formation and nitrogenase activity are derepressed in the mutant.

Strain SA-1 failed to grow in medium containing L-glutamate or L-aspartate as the nitrogen source, although the parent strain (SA-0) grew well utilizing these compounds as the sole N source. This defect is probably at the level of transport, since strain SA-1 incorporated [^{14}C]glutamate at less than 15% of the parent value (0.13 U [1U is 1 μmol · day^{-1} · mg of dry weight^{-1}] compared with 0.9 U for the parent). L-aspartate was incorporated by strain SA-1 at about 32% of the parent strain value (0.38 U). Both strains assimilated glutamine well (0.63 and 0.54 U for strains SA-O and SA-1, respectively). These results show that resistance to MSX also affected the transport of glutamate and aspartate, which is similar to the observations made by Chapman and Meeks (6) for *A. variabilis*.

Kinetic properties of glutamine synthetase from mutant strain SA-1. Since glutamine synthetase is the primary target of MSX, the MSX-resistant strain SA-1 produced a glutamine synthetase that was insensitive to this analog (Fig. 2). MSX, at a concentration as high as 200 μM, inhibited glutamine synthetase biosynthetic activity by less than 15%,

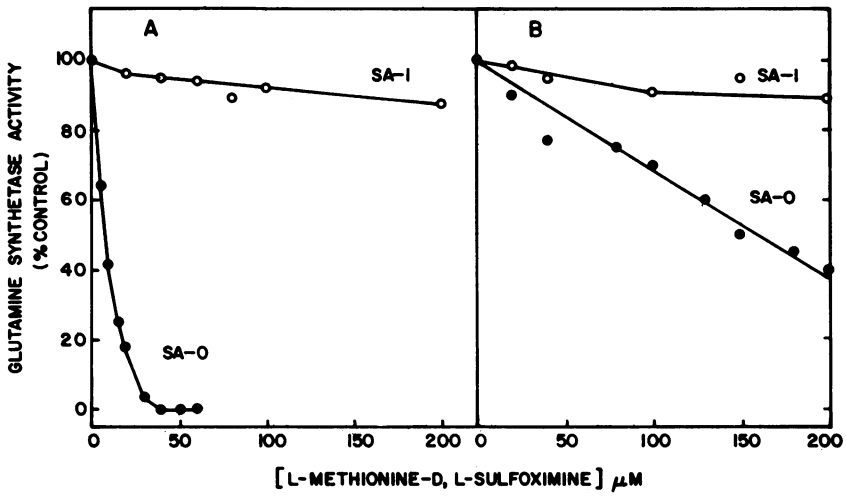

FIG. 2. Effect of the L-glutamate analog MSX on glutamine synthetase activity from the parent (SA-0) and the MSX-resistant mutant (SA-1). (A) Biosynthetic activity; (B) γ-glutamyltransferase activity.

while the glutamine synthetase from the parent was completely inhibited by less than a 40 μM concentration (Fig. 2A). The γ-glutamyltransferase activity of glutamine synthetase from the mutant was also insensitive to MSX (Fig. 2B). Although the enzyme showed differences in the level of inhibition of MSX, the apparent K_m values for glutamate, glutamine, and hydroxylamine determined with crude extracts were similar for the enzymes from the parent and mutant (data not shown). Because of the lower specific activity of the mutant enzyme in the extracts (Table 5), the apparent K_m for NH_4^+ was not determined, but this value, based on estimation, was not too dissimilar to the value observed with the enzyme from the parent. Glutamine synthetase activity from the parent and the mutant had similar temperature and pH profiles. These results show that the mutation leading to MSX resistance altered the biochemical properties of glutamine synthetase in strain SA-1.

DISCUSSION

Derepression of nitrogenase synthesis in the presence of NH_4^+ can be accomplished by genetic modification of the *ntr* and *nif* genes (3). Derepression of nitrogenase synthesis can also be achieved by blocking the rate of NH_4^+ assimilation in the cell, either by a mutation in glutamine synthetase or glutamate synthase or both or by adding inhibitors (MSX) to abolish glutamine synthetase catalytic activity (31). Addition of other amino acid analogs (7-azatryptophan, β-hydroxynorvaline) to the growth medium also derepresses nitrogenase synthesis in the presence of NH_4^+ (13, 38). Production of nitrogenase in an NH_4^+ assimilation-defective mutant, even in the presence of high external concentrations of NH_4^+, is due to a physiological effect of low glutamate pool levels (29). Although mutant strains of this class can express several other enzyme systems producing NH_4^+ or glutamate (for example nitrate assimilation, amino acid utilization, etc.) or both in the presence of NH_4^+, these operons can be repressed by the presence of amino acids in the medium (31). Mutant strains altered in the rate of NH_4^+ assimilation do excrete the NH_4^+ produced by nitrogenase into the medium. In this regard, mutant strains of this type resemble symbiotic nitrogen-fixing organisms (19, 21, 25) and can be recognized and isolated in weakly buffered media containing pH indicator dyes. Terzaghi (40) used pH indicators to identify *Azotobacter* mutants that were derepressed for nitrogenase synthesis in the presence of NH_4^+. In this medium, the disappearance of H^+ as H_2 (catalyzed by nitrogenase) was monitored. The pH indicator medium used in this study was designed to isolate mutant strains that excrete NH_4^+, because the H_2 produced by the enzyme was very effectively reutilitized by heterocysts.

Mutant strains that are resistant to the glutamate analog MSX can be readily isolated (6, 34, 37). Some of the mutant strains were also derepressed for nitrogenase synthesis in the presence of NH_4^+ (Table 1). Although most of the MSX-resistant mutants were capable of excreting at least some NH_4^+ into the medium (34; Table 2), not all of these mutants were derepressed for nitrogenase synthesis in the presence of NH_4^+. This is probably a reflection of the rate of NH_4^+ assimilation in these organisms, a property that is similar to the observations made on the initial group of nitrogenase-derepressed mutants of *K. pneumoniae* (29). An indirect correlation between the rate of NH_4^+ assimilation and the extent of derepression of nitrogenase synthesis exists in these organisms.

Strain SA-4 excreted NH_4^+ at low rates and assimilated most of the NH_4^+ produced from N_2 into cell material (Tables 2 and 3). This strain produced nitrogenase activity at higher levels than did the parent culture (greater than twofold) and also derepressed the synthesis of nitrogenase in the presence of NH_4^+. However, in contrast to other nitrogenase-derepressed mutants (strains SA-1, SA-6, etc.), this derepression in strain SA-4 did not extend to the nitrate assimilation pathway (Table 4). These results suggest that strain SA-4 is affected only in the regulation of nitrogen fixation and is different from other mutants like strain SA-1, which carry defects in NH_4^+ assimilation. Additional experiments are needed to determine the biochemical and genetic alteration(s) in this strain.

Even in derepressed mutants, addition of NH_4^+ and glutamine to the filaments during the assay led to a decrease in the specific activity of nitrogenase (Table 1). This is in general agreement with the observations made by Turpin et al. (41) that addition of NH_4^+ inhibited nitrogenase activity in *A. flos-aquae* and this inhibition could be reversed by MSX. This decline in nitrogenase activity may be a consequence of the partitioning of the reductant between the growth of vegetative cells and nitrogen fixation in heterocysts. It is interesting to note that some of the mutant strains failed to grow even in the presence of NH_4^+ (Table 3). Mutant strains with similar phenotype have been described in *Anabaena* sp., and the biochemical defects in these mutants are unknown (9). The *A. variabilis* mutants (strains SA-5 and SA-6) also produced and accumulated large amounts of nitrogenase-produced NH_4^+ into the medium (Table 2), and this may be the cause of the low growth rate of the mutants under N_2-fixing conditions.

The defect in NH_4^+ assimilation and growth could be a result of kinetic alteration of glutamine synthetase introduced by the mutation (Tables 2 and 3). However, no detectable differences in the apparent K_m values could be observed between the glutamine synthetases from the parent and at least one mutant (strain SA-1) studied in detail.

Papan and Bothe (24) observed that glutamine synthetase from *A. cylindrica* was activated by thioredoxin. It is likely that glutamine synthetase from the mutant strain SA-1 is not activatable by this activation system and thus produced low activity (Table 5). Additional experiments with pure glutamine synthetase from the parent and mutant are necessary to establish the biochemical alteration of the enzyme in the mutant.

In several microorganisms, the syntheses of nitrogenase, nitrate reductase, and nitrite reductase activities are repressed by ammonium ion (23, 29). In *A. variabilis*, the synthesis of nitrogenase was found to be insensitive to nitrate (15 mM) in the medium. This may be a consequence of the low levels of nitrate reductase and nitrite reductase activities detected in the cell. MSX-resistant mutants produced these enzymes constitutively, while the parent strain did require nitrate for expression of the two enzyme activities. Another unique aspect of nitrate and nitrite reductase activities in this organism is their insensitivity to the presence of ammonia in the growth medium, although addition of ammonia to the assay inhibited nitrate reductase activity. These results clearly demonstrate that the mutation leading to derepression of nitrogenase has pleiotropic effects on other ammonia-producing enzyme systems.

Photoproduction of NH_4^+ by cyanobacteria has been studied in detail with MSX used to derepress nitrogenase in the presence of NH_4^+ (18, 26). The availability of mutant strains overcomes the limitation of continual addition of a toxin, MSX, to maintain NH_4^+ production and excretion. These mutant strains can potentially serve as an N fertilizer

supplier and support plant growth under N-limiting conditions. Cyanobacteria are ubiquitous and utilize sunlight and water for NH_4^+ production and are implicated in enhancement of soil fertility. In several parts of the world, cyanobacteria are applied to croplands, especially rice paddies, as fertilizer supplement. But naturally occurring, wild-type, free-living cyanobacteria assimilate all the NH_4^+ produced by nitrogenase for their own growth, and the plant obtains this nitrogen only after the death and decay of the cyanobacteria. In this regard, a cyanobacterial mutant capable of exporting fixed N_2 as NH_4^+ may potentially provide nitrogen to the standing crop.

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