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Mutants of *Anabaena* Strain CA Altered in Their Ability to Grow Under Nitrogen-Fixing Conditions

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Mutants of *Anabaena* strain CA impaired in nitrogenase activity and growth on N_2 were isolated and characterized. One mutant was selected for resistance to L-methionine-DL-sulfoximine, and others were selected for resistance to DL-7-azatryptophan or for requirements for combined nitrogen. The mutants varied in sensitivity of growth and nitrogenase activity to atmospheric O_2. Several of the mutants whose growth on N_2 was impaired under aerobic conditions could grow and reduce acetylene at rates comparable to the wild type when grown microaerobically under N_2-CO_2 (99:1). The acetylene reduction activity of some of the strains grown under N_2-CO_2 was immediately and completely lost upon exposure to atmospheric O_2, but in at least one strain this loss was reversed when the O_2 concentration was lowered, even after 10 h of exposure to air. The characteristics of the O_2-sensitive mutants suggest that there may be several sites sensitive to O_2 and that the protective mechanism involves several different phenomena.

The importance of nitrogen fixation by members of the cyanobacteria (blue-green algae) as a significant component of the global nitrogen cycle is now well recognized. In natural ecosystems, these organisms can contribute more fixed nitrogen than other free-living microbes (26). They are also recognized as being largely responsible for the fertility of certain important agricultural systems, such as tropical rice paddy soils (33). An attractive feature of cyanobacterial nitrogen fixation is that its demands for energy and reductant are supplied by the photosynthetic activity of the cells. Thus, the process is essentially light dependent and requires no external supply of preformed organic compounds. The filamentous nitrogen-fixing cyanobacteria are also of interest as model systems for the study of cellular differentiation. Specialized cells known as heterocysts are produced in response to a deficiency in combined nitrogen in the environment (7). The heterocysts are believed to be the major site of nitrogen fixation under aerobic conditions and possess a number of morphological and physiological specializations for this purpose (6, 10, 34, 35).

Nevertheless, nitrogen fixation has been most thoroughly studied in the agriculturally important *Rhizobium*-legume associations and in the heterotrophic bacteria *Klebsiella*, *Azotobacter*, and *Clostridium* (4, 5, 9, 15, 27). Studies of the genetics of nitrogen fixation by analysis of mutant strains, especially in *Klebsiella pneumoniae*, have yielded a wealth of information on the physiology, biochemistry, and regulation of the system and have revealed its considerable complexity (1, 17, 18, 31, 32). Currently there exists no comparable information concerning the cyanobacteria. The first step toward obtaining such information must be the isolation of mutant strains altered in their ability to utilize N_2 or in their regulatory properties. A number of such strains have been produced (3, 19-21); however, the lesions have not been biochemically defined, and in most cases the phenotypes have been only partially characterized. Several of the mutants isolated by Haury and Wolk (11) were capable of nitrogenase activity only under microaerobic conditions or anaerobic conditions with added dithionite. The authors suggested that the latter represented mutants impaired in electron transport to nitrogenase.

In this paper we report the isolation of mutants altered in nitrogen fixation from the filamentous cyanobacterium *Anabaena* strain CA, after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Mutants were selected as strains resistant to the inhibitors L-methionine-DL-sulfoximine (MSX) and DL-7-azatryptophan (AZA-T) or as strains requiring combined nitrogen for normal growth. The mutants were characterized by their growth on various nitrogen sources and by sensitivity of growth and nitrogenase activity to atmospheric oxygen. Several of the mutants displayed a marked sensitivity to

O_2 and promise to be of value for investigations concerning the O_2 protection mechanism of this organism.

MATERIALS AND METHODS

Organism and culture conditions. *Anabaena* strain CA (ATCC 33047), a rapidly growing filamentous cyanobacterium, was the parent strain used. Growth experiments were carried out at 39°C under the culture conditions previously described for this organism (23). For microaerobic growth, culture tubes were sealed with washed rubber stoppers through which a cotton-plugged sparging tube and vent tube allowed continuous gassing of N_2-CO_2 (99:1). Growth rates of the microaerophilic cultures were measured after the second transfer under the described conditions. Growth was measured turbidimetrically with a Lumetron model 402E colorimeter with a colored-glass filter set with peak transmission at 660 nm. Growth was expressed as generation times (hours) or as the specific growth constant (*k*) in $\log_{10}$ units per day (13).

Reagents. The compounds NTG, MSX, and AZA-T were purchased from Sigma Chemical Co., St. Louis, Mo. Solutions of these compounds were filter sterilized before use. All other compounds were of reagent grade.

Mutagenesis and selection procedures. In each mutation experiment, a 20-ml culture of *Anabaena* strain CA was grown to an optical density of about 0.22 (0.12 mg [dry weight] per ml). The cell suspension was then incubated in the dark at 39°C (with continuous gassing of air plus 1% CO_2) for 12 to 16 h, to deplete endogenous metabolite pools. After this dark pretreatment, a freshly prepared solution of NTG was added to 10 ml of the cell suspension (final concentration of NTG, 40 μg/ml), and the suspension was then kept in the dark at room temperature for 30 min. The cells were washed free of NTG by two cycles of centrifugation and suspension in sterile growth medium minus combined nitrogen. The filaments were then broken into single cells by a gentle sonication (four 1-s bursts at output setting 4, Sonifier model S 125; Branson Instruments Co., Stamford, Conn.) with aseptic precautions. This procedure yielded at least 80 to 90% intact single cells in the suspension. A series of dilutions of the resulting suspension was immediately plated on growth medium solidified with 1.0% agar (0140; Difco Laboratories, Detroit, Mich.). Selective plates were made containing 5.5 μM MSX or 80 μM AZA-T in media with the normal 12.6 mM $NaNO_3$. Other plates were made containing low levels of $NaNO_3$ (60 μM) or L-glutamine (34, μM). The plates were incubated at 39°C in sealed GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) containing air enriched with 2.0% CO_2. The jars were suspended in water baths identical to those used for growth of liquid cultures. Under these conditions, wild-type cells grew to large (2-mm), distinct colonies in 3 to 4 days. Putative mutants were picked from the plates and restreaked onto selective plates at least twice to be certain of obtaining clones.

Nitrogenase activity. The acetylene reduction assay (28) was used to measure nitrogenase activity. Samples (2.0 ml each) of growing cultures were placed in 8-ml Vacutainer tubes (Becton, Dickinson & Co., Rutherford, N.J.) and sealed with washed serum stoppers. An atmosphere of argon was provided by flushing the assay tubes for about 5 min; the cells were then added, and the gas was bubbled through the suspension for an additional 3 min. CO_2 was then injected to 5% of the gas phase, and C_2H_2 was injected to 10%. For microaerobically grown cells, the samples were taken without exposing the cells to air; after we added the suspension to pregassed assay tubes, the argon was bubbled through for 1 min before addition of CO_2 and C_2H_2. Assays were also performed under an atmosphere of air plus 5% CO_2 and 10% C_2H_2. The assays were incubated, and ethylene production was measured as described elsewhere (J. W. Gotto, F. R. Tabita, and C. Van Baalen, Arch. Microbiol., in press). Whole-cell protein was determined by the method of Lowry et al. (14) after digestion of the samples for 90 min in 0.5 N NaOH at 50°C. Bovine serum albumin was used as a reference standard.

Oxygen measurements. O_2 was measured with a Clark-type electrode (no. 5331; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) mounted in a water-jacketed cell (no. OX 15253; Gilson Medical Electronics, Inc., Middleton, Wis.). Changes in electrode current were detected and amplified on a Keithly model 150B microvolt-ammeter. All measurements were made at the growth temperature, 39°C. Measurements of microaerobically grown cell suspensions were made by sampling the suspensions with a syringe in such a way that no air was admitted and by adding the sample to the electrode chamber, which had previously been flushed with N_2.

RESULTS

Selection of mutants. Mutant strains of *Anabaena* strain CA were easily discerned after NTG treatments. Strains resistant to MSX or AZA-T were selected as large, apparently healthy colonies, growing on plates containing inhibitor concentrations (5.5 μM MSX or 80 μM AZA-T) which were lethal to the wild type. Other mutants (JM1, JM4, and JM5) were selected as small or light-green to yellow colonies, whose growth was presumably limited by the concentration of combined nitrogen, on plates containing low amounts of $NaNO_3$ or L-glutamine. The phenotypic characteristics of the mutant strains thus obtained have proven to be quite stable and reproducible.

Growth characteristics. Strain PM10 was selected for MSX resistance. The growth response of this strain to increasing amounts of this inhibitor is shown in Fig. 1. Strains GM4, GM5, and GM9 were selected for AZA-T resistance; Fig. 2 shows their growth response to increasing amounts of AZA-T. Growth of wild-type strain CA was inhibited by 5 μM MSX or 40 μM AZA-T. These strains clearly differ from each other in their degree of resistance. Figure 2 also shows that PM10, although selected for MSX resistance, is also resistant to AZA-T.

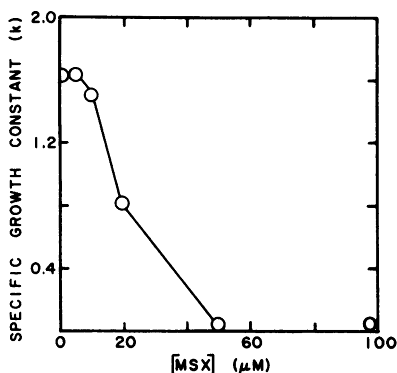

FIG. 1. *Growth of mutant strain PM10 as a function of MSX concentration in a medium containing 12.6 mM $NaNO_3$. Growth rates are expressed in terms of the specific growth constant (k) in log_{10} units per day.*

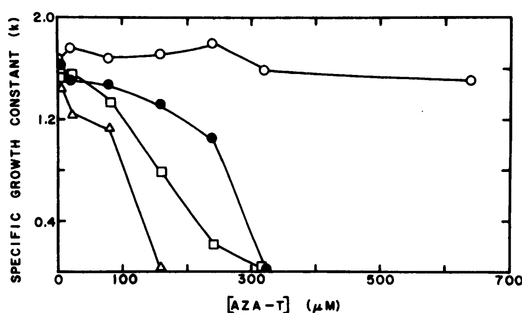

FIG. 2. *Growth of mutant strains as a function of AZA-T concentration in a medium containing 12.6 mM $NaNO_3$. Symbols: ○, GM9; □, GM5; △, GM4; ●, PM10. Growth rates are expressed in terms of the specific growth constant (k) in log_{10} units per day.*

None of the AZA-T-resistant strains, however, is resistant to MSX as well.

The growth characteristics of the mutants with respect to the nitrogen source are shown in Table 1. It is evident that, although growth on combined nitrogen is little affected in most cases, aerobic growth on N_2 is severely limited. The C_2H_2 reduction values of the strains which grew slowly on N_2 (aerobically) showed a marked decrease in activity when assayed in air, as opposed to assays under argon (Table 2). This suggested the possibility of damage to the supposed mechanism which protects nitrogenase from inactivation by O_2. All of the strains were accordingly screened for microaerophilic growth under N_2-CO_2. Several of the strains (GM5, GM9, and JM5) showed a dramatic response to these conditions, with growth rates approaching that of the wild type (Table 1). Strains GM4 and JM1, however, still grew poorly and ceased growth at low cell densities, possibly because of O_2 buildup in the culture.

Nitrogenase activity. The acetylene-reducing activities of the mutants, grown aerobically or microaerobically, are shown in Table 2. In agreement with the growth data, strains GM9 and JM5 possess high levels of nitrogenase activity when grown under N_2-CO_2. This activity, however, is completely and immediately lost upon exposure to atmospheric levels of O_2. This indicates that these strains have, in fact, lost

TABLE 1. *Growth of mutant and parent strains on various nitrogen sources*[a]

Strain	Generation time (h)			
	N_2	2 mM NH_4NO_3	12.6 mM $NaNO_3$	N_2 (microaerobic)
CA (wild type)	4.7	3.8	4.3	5.0
GM4	NG[b]	4.9	5.0	15.6[c]
GM5	17.3	4.2	4.8	5.7
GM9	12.6[c]	4.2	4.3	5.6
JM1	NG	5.5	6.8	11.0[c]
JM4	13.4	5.4	7.1	9.3
JM5	17.5[c]	4.0	5.0	8.5
PM10	12.4	3.9	4.4	7.5

[a] Values represent the means of three experiments. Growth conditions were as described in the text.

[b] NG, No growth.

[c] Growth occurred at the indicated rate, but ceased when the optical density of the culture reached approximately 0.2 (about 0.11 mg [dry weight] per ml). All others maintained the indicated rates beyond an optical density of 1.0.

TABLE 2. *Acetylene-reducing activity of growing cultures of mutant strains*[a]

Strain	nmol of C_2H_4 (mg of protein × min)$^{-1}$			
	Aerobically grown cells		Microaerobically grown cells	
	Argon-CO_2 assay	Air-CO_2 assay	Argon-CO_2 assay	Air-CO_2 assay
CA (wild type)	39.8	36.8	35.2	32.4
GM4	—[b]	—	2.9	0
GM5	9.2	5.7	24.0	2.0
GM9	—	—	32.0	0
JM1	—	—	4.0	0
JM4	10.9	0.6	22.2	0.3
JM5	—	—	22.9	0
PM10	23.4	8.7	ND[c]	ND

[a] The medium contained no combined nitrogen. Assay procedures are described in the text. Values represent the average of duplicate determinations. Assays were performed when the cell density reached 0.10 to 0.13 mg (dry weight) per ml.

[b] —, Mutant will not grow under this condition.

[c] ND, Not determined.

their ability to protect nitrogenase from O_2. Strains GM5 and JM4 also showed considerable nitrogenase activity when grown microaerobically. Although this activity was greatly decreased upon exposure to O_2, these strains did retain slight activity under aerobic conditions, and, in fact, they were able to maintain slow growth rates under aerobic nitrogen-fixing conditions (Table 1). The failure of strains GM4 and JM1 to display high nitrogenase activity under any conditions tested may reflect a more extreme O_2 sensitivity, but may also be due to lesions in other aspects of the nitrogen fixation system.

The O_2 sensitivity of strain GM9 was examined further. Although the nitrogenase activity of microaerobically grown cells was lost immediately upon exposure to air, there appeared to be no irreversible damage to the enzyme. Table 3 shows that even after 10 h of exposure to atmospheric O_2, full nitrogenase activity could be restored simply by gassing the suspension with argon for 1 min. Electrode measurements indicated that 1 min of argon gassing lowered the O_2 concentration in the suspension to 0.33 μl/ml. At the same cell density, the O_2 concentration in the microaerobically grown suspension, before exposure to air, was 0.24 μl/ml. An air-saturated suspension at this temperature had an O_2 concentration of 4.40 μl/ml.

DISCUSSION

The mutant strains described above represent a variety of phenotypes altered in their capacity to utilize molecular nitrogen. Their ease of isolation from strain CA is reminiscent of the ease of induction via NTG of a variety of nitrate reductase pathway mutants in other cyanobacteria (24, 25). However, a relationship between these two general types of nitrogen assimilation mutants is not implied by the data at hand. The most striking characteristic of these Nif-impaired mutants observed thus far is the varied sensitivity of their nitrogen-fixing activities to O_2, in comparison with the parent strain, which is scarcely affected. The nitrogenase of cyanobacteria and other organisms in vitro is known to be irreversibly inactivated by exposure to oxygen (2, 12), and thus some mechanism must function to protect the enzyme from O_2 in vivo. Clearly, the O_2 sensitivity of the above strains suggests mutations in the protective mechanism. In this connection, it is interesting to note that even the very sensitive strains, GM9 and JM5, when grown microaerobically, produced heterocysts which appeared morphologically normal under a light microscope, at a frequency comparable to the wild type. However, this does not rule out the possibility of ultrastructural changes. Strains GM5, JM4, and PM10 also produce heterocysts with no obvious morphological abnormalities. Strains GM4 and JM1, which were unable to grow on N_2 aerobically and which grew only slightly on N_2 microaerobically, produced heterocysts which appeared highly disorganized and aberrant, but at a normal frequency. At this time it is not known whether nitrogenase activity in these two strains could be increased by a more rigorous exclusion of O_2 or whether their lack of activity reflects damage to some other component of the nitrogen-fixing system.

The other strains (GM5, GM9, JM4, JM5, and PM10) seem to represent a gradation of sensitivity to O_2, as suggested by their different responses to atmospheric O_2 in terms of growth and nitrogenase activity. These mutants were selected for different characteristics and are obviously different from each other phenotypically. If they are, in fact, different genotypes, the fact that they all display some degree of O_2 sensitivity would imply that the mechanism of protection from O_2 is quite complex and involves at least several different phenomena.

The rapid (within minutes) reversibility of O_2 inhibition of nitrogenase activity demonstrated in strain GM9 is a phenomenon which has not been reported in the cyanobacteria. Like strain GM9, the non-heterocystous *Plectonema boryanum* could grow and express nitrogenase activity in the absence of combined nitrogen under microaerobic conditions (29); however, in *Plectonema*, the loss of in vivo activity after 15 min of exposure to an atmospheric level of O_2 was not reversible. Rippka and Stanier (16) studied the induction of nitrogenase in different strains of *Anabaena* under anaerobic conditions and found that the loss of nitrogenase activity after

TABLE 3. *Reversibility of O_2 inhibition of nitrogenase activity in mutant strain GM9*[a]

Length of exposure to air-CO_2	nmol of C_2H_4 (mg of protein × min)$^{-1}$	
	Argon-CO_2 assay	Air-CO_2 assay
10 min	24.3	0
4 h	25.6	1.0
10 h	30.4	1.7

[a] GM9 was grown microaerobically, as described in the text, in a medium lacking combined nitrogen to an optical density of 0.20. The culture was then gassed with air plus 1% CO_2 while remaining under growth conditions, and C_2H_2 reduction assays were performed on samples taken at the indicated times. The argon-CO_2 assays were performed after argon had been bubbled through the samples for 1 min.

1 h of exposure to an atmospheric level of O_2 was complete and irreversible. Thus, it appears that in the mutant GM9, nitrogenase itself must still be protected in some way from damage by O_2, since its full activity can be restored by simply lowering the O_2 concentration to 7.5% of the air-saturated level. This suggests that at least one oxygen-sensitive mechanism in cyanobacteria involves a rapidly reversible component, possibly related to electron transport and not, apparently, directly to nitrogenase.

The relationship between resistance to MSX or AZA-T and oxygen-sensitive nitrogenase activity is not clear. The effect of MSX in relieving the ammonia repression of heterocyst and nitrogenase synthesis is thought to be due to its inhibition of glutamine synthetase (8, 30). MSX resistance could also have an effect on the regulation of glutamine synthetase which in some way relates to O_2 sensitivity. The site of action of AZA-T is not known, but seems to be separate from that of MSX (22). Again, it is hard to visualize a connection between AZA-T and O_2 sensitivity, except possibly through some feature of tryptophan metabolism related to nitrogen fixation. The glutamine synthetase activity of the MSX-resistant strain PM10 was not lower than that of the wild type, and the AZA-T-resistant strains also had glutamine synthetase activity comparable to that of the wild type (unpublished data). Haury and Wolk (11) have suggested altered heterocyst lipids as a basis for O_2 sensitivity in certain Nif^- mutants of *Anabaena variabilis*. Although we have not examined this point in the strain CA mutants, it is difficult to imagine both AZA-T and MSX resistance correlating with heterocyst lipids. In addition, none of the other O_2-sensitive strains was found to be resistant to either inhibitor. Further analysis of these mutants will undoubtedly be helpful in revealing the properties of the nitrogen fixation system of the cyanobacteria.

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