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Classes of *Anabaena variabilis* Mutants with Oxygen-Sensitive Nitrogenase Activity

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Mutants of *Anabaena variabilis* deficient in the envelope glycolipids of heterocysts have no or very low nitrogenase activity when assayed aerobically. Revertants capable of aerobic growth on N_2 have increased quantities of these glycolipids. Among mutants which require fixed nitrogen for growth in air and which have a normal complement of glycolipids, one expresses high nitrogenase activity at low oxygen tension. Three others show high nitrogenase activity only in the presence of dithionite and are therefore impaired in electron transfer.

Nitrogenase functions in many photosynthetic, i.e., O_2-evolving, cyanobacteria (blue-green algae) even though the enzyme is highly O_2 labile. In certain of these organisms, protection from inactivation by O_2 is related to the occurrence of differentiated cells called heterocysts, in which nitrogenase is completely or nearly completely localized during aerobic growth (3, 10, 17, 19, 21, 22; R. B. Peterson and C. P. Wolk, Abstr. 3rd Int. Symp. N_2 Fixation, p. 18). The isolation of mutants of *Anabaena variabilis* Kütz. requiring nitrate or ammonium for growth in air (2) offered an opportunity to elucidate the physiological systems supporting nitrogen fixation in a heterocyst-forming cyanobacterium. In this paper, we identify certain of these mutants as being capable of nitrogenase activity, but only under microaerobic or anaerobic conditions.

MATERIALS AND METHODS

The mutant strains of *A. variabilis* Kütz. (ATCC 29413) examined in the series of experiments to be described were isolated, after mutagenesis with nitrosoguanidine and enrichment with penicillin, as strains requiring fixed nitrogen (either ammonium or nitrate will suffice) for growth (2). All strains were grown with 2.5 mM $NaNO_3$ and 2.5 mM KNO_3 on culture medium (1) which was either solidified with 1% (wt/vol) purified agar (2) or, for liquid culture, diluted eightfold (AA/8). Liquid cultures (25 ml in 50-ml flasks) were grown at room temperature (ca. 23°C) at 88 rpm on a rotatory shaker (model 6140; Eberbach Corp., Ann Arbor, Mich.). The light intensity at 85 cm from four cool-white fluorescent lamps (Sylvania FR40CW-235) was approximately 4,900 erg $cm^{-2}s^{-1}$ as measured with a model 68 Kettering Radiant Power Meter (Laboratory Data Control, Riviera Beach, Fla.) and 280 foot-candles as measured with a type 214 light meter (General Electric Co., Cleveland, Ohio). For assays of glutamine synthetase activity (see below), cultures having a volume of up to 400 ml were employed.

Wild type and mutants grown with nitrate to 1.4 to 6.2 μg of chlorophyll *a* per ml (8) were washed free of nitrate by two cycles of centrifugation (5 min, 250 × *g*) and suspension in 20 ml of AA/8 and were finally suspended in AA/8 at a density of 1 μg of chlorophyll *a* per ml. Portions (35 ml) were placed into stoppered, sterile glass test tubes (25 by 150 mm). Each tube was bubbled with gas (see below) at ca. 40 ml/min, which helped to keep the filaments dispersed. The gas was sterilized by passage through a sterile 0.22-μm-pore size membrane filter (Millipore Corp., Bedford, Mass.) and passed through a 2-mm-ID glass tube which extended to within 1 cm of the bottom of the test tube. Also to aid dispersion of the filaments, the test tubes, mounted at 45° from the vertical, were rotated at 125 rpm on a model 6140 shaker (Eberbach) within a growth chamber (model MG-8; Sherer-Gillett Co., Marshall, Mich.) maintained at 30°C. Illumination was provided by two cool-white fluorescent lamps (Sylvania FR40CW-235) 38 cm above the test tubes. The light intensity was ca. 5,400 erg $cm^{-2}s^{-1}$.

After 4 days of bubbling with humidified CO_2 in N_2 (1:99, vol/vol; a mixture of gasses purchased individually from Matheson Gas Products, Joliet, Ill.), the cultures were sampled under anaerobic conditions. Portions of the cultures were supplemented with 4 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; Sigma Chemical Co., St. Louis, Mo.), pH 7.6. Acetylene reduction by the intact filaments was assayed in the light in 5-ml serum vials under an atmosphere of 0.3 cm^3 of acetylene in argon (22) after a 30-min incubation in the absence or presence of 5 mM $Na_2S_2O_4$. Alternatively, the cultures were gassed with humidified air for 4 days, and acetylene reduction was assayed in the light under 0.3 cm^3 of acetylene in air. Protein determinations (7) were made on samples which were frozen, thawed, and cavitated in a test tube held for 8 min in a sonic cleaning bath (model 8845-3; Cole-Parmer Instrument Co., Chicago, Ill.). Cultures of mutants were also bubbled with CO_2 in N_2 for 4 days, followed by air for 2

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days, and acetylene reduction activity was then measured aerobically.

Glutamine synthetase activity was assayed (13) by using material grown with AA/8 plus nitrate, washed (see above), incubated in AA/8 for 4 to 5 days, and then cavitated at 13°C in a Branson model S-125 Sonifier (Heat Systems Co., Melville, N.Y.) under air at 2.5 A for 60 s/ml. This cavitation breaks virtually all cells. For determination of the localization of tetrazolium reduction (17), suspensions of filaments which had been incubated in AA/8 for from 4 to 6 days under air were evacuated and regassed with H_2. They were then combined anaerobically with an equal volume of a 0.1% (wt/vol) solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) in 0.1 M TES, pH 7.2, under H_2 and were incubated in the dark at room temperature. Samples were taken at intervals and examined by light microscopy for the production of formazan, the product of reduction of 2,3,5-triphenyltetrazolium chloride.

For determination of heterocyst lipids, wild-type and mutant cultures were washed free of nitrate as described above, suspended in 100 ml of AA/8 in 250-ml Erlenmeyer flasks, and incubated for 6 days to permit the differentiation of heterocysts. The development of heterocysts was followed by light microscopy, and heterocysts were enumerated with a hemocytometer. Lipids were extracted with chloroform-methanol (2:1, vol/vol) from pellets (5 min, 250 × *g*) of filament suspensions containing 3.7×10^7 heterocysts. Published methods were used for chromatography (9), visualization (9), and identification of the lipids (4, 6). To select for revertants able to grow aerobically on N_2, mutants were washed free of nitrate (see above), cavitated to fragment the filaments (2), and incubated in the light on nitrate-free, solidified culture medium.

RESULTS

In vivo acetylene reduction (nitrogenase activity) by filaments of cyanobacteria can be limited by the rate of supply of reductant and ATP to the protein constituents of nitrogenase as well as by the amounts of those protein constituents present and active, amounts which in turn depend upon the extent of the protection of those constituents from inactivation by oxygen. A group of mutants incapable of utilizing dinitrogen for growth in air (2) was surveyed to identify strains defective either in protection of nitrogenase from oxygen or in provision of reductant to the enzyme.

Table 1 shows the mean acetylene-reducing activity of wild-type and mutant cultures in AA/8 after 4 days of bubbling with air or N_2–CO_2 or after 4 days with N_2–CO_2 and 2 days with air. Samples from cultures bubbled with air were assayed aerobically, whereas samples from cultures incubated with N_2–CO_2 were assayed microaerobically and, by the addition of dithionite, anaerobically. Dithionite also serves as a source of reductant to cyanobacterial nitrogenase (5, 10, 17, 19).

The acetylene-reducing activity of wild-type cultures was greatest under argon and was decreased approximately 26% by the presence of dithionite; the activity was lowest when measured in air after a period of aerobic incubation. The average rate of reduction of acetylene by mutant NF-77 was more than 50% of the wild-type rate under microaerobic conditions, but

TABLE 1. *Mean acetylene-reducing activity of wild-type and mutant strains of A. variabilis*

Strain	Acetylene-reducing activity (nmol per mg of protein per min) under the following conditions of assay[a]:		
	Air	Microaerobic	Anaerobic with $Na_2S_2O_4$
Wild type	2.5 ± 1.2[b]	6.2 ± 1.8	4.6 ± 1.3
Mutants active in acetylene reduction under microaerobic conditions; glycolipids normal			
NF-77	0.2 ± 0.1[b]	3.3 ± 0.7	4.0 ± 0.3
Mutants active in acetylene reduction in the presence of dithionite; glycolipids normal			
NF-12	0	0.6 ± 0.2	3.2 ± 1.0
62	0	0.4 ± 0.1	2.0 ± 0.5
63	0	0.3 ± 0.1	2.1 ± 0.8
Mutants deficient in heterocyst glycolipids I and III			
NF-3	0	0.2 ± 0.1	1.0 ± 0.6
53	0	1.1 ± 0.7	3.9 ± 2.0

[a] Wild-type and mutant strains of *A. variabilis* were incubated microaerobically (unless otherwise indicated) for 4 days and then assayed either under air or under argon in the absence or presence of 5 mM $Na_2S_2O_4$.

[b] Incubated under air for 4 days. Similar results were obtained if the cultures were first incubated microaerobically for 4 days and then incubated aerobically for 2 days.

only 8% of the wild-type activity in air. The nitrogenase activities of NF-77 and the other mutants, unlike the activity of wild-type cultures, were greater under anaerobic than under microaerobic conditions. Three mutants, NF-12, 62, and 63, reduced acetylene anaerobically in the presence of dithionite at 44 to 70% of the rate of wild-type cultures, but at only 5 to 10% of the rate of wild-type cultures under microaerobic conditions and did not reduce acetylene in the presence of air. Mutants NF-3, 15, 35, 47, 53, and 58, the heterocysts of which are deficient in their content of envelope glycolipids (see below), had no or very low nitrogenase activity (<0.1 nmol of C_2H_4 per mg of protein per min) when assayed under air and measurable but very variable activity when assayed microaerobically or anaerobically after microaerobic incubation.

Wild-type *A. variabilis* incubated with 2,3,5-triphenyltetrazolium chloride under H_2 in the dark deposited crystals of formazan in heterocysts within 15 to 20 min and in a low percentage of vegetative cells within 1 h. Under these conditions, all heterocysts of mutants NF-62 and 63 showed crystals of formazan within 25 min, and a majority of heterocysts of NF-12 showed crystals of formazan within 40 min.

The mutants shown in Table 1 all grow on nitrate at approximately the same rate as does wild-type *A. variabilis*, and all produce heterocysts in AA/8 medium. Vegetative cells of NF-12 (2) and NF-20 differentiate into heterocysts at an abnormally high frequency, and vegetative cells of NF-76 differentiate at a very low frequency (2) when plated on agar at high density. The specific glutamine synthetase activity (mean of three determinations) of mutants NF-12, 20, and 76 was 5.5, 5.3, and 6.9 nmol of γ-glutamyl hydroxamate formed per mg of protein per min, respectively, or 76 to 99% of the activity of the wild-type organisms.

The content of the glycolipids comprising the inner layer of the envelope of heterocysts (20) in NF-77, 12, 62, and 63 was similar to the content of those glycolipids in wild-type *A. variabilis*. However, there was a marked deficiency of those lipids in mutants NF-3, 15, 47, 53, and 58 (Fig. 1 and 2). Mutants NF-15, 47, and 58 gave rise to spontaneous revertants which were capable of N_2 fixation under aerobic conditions and all of which had an increased amount of those glycolipids (Fig. 2), whereas mutants NF-3 and 53 have not yielded stable revertants. Each sample represents an extract of 3×10^7 heterocysts.

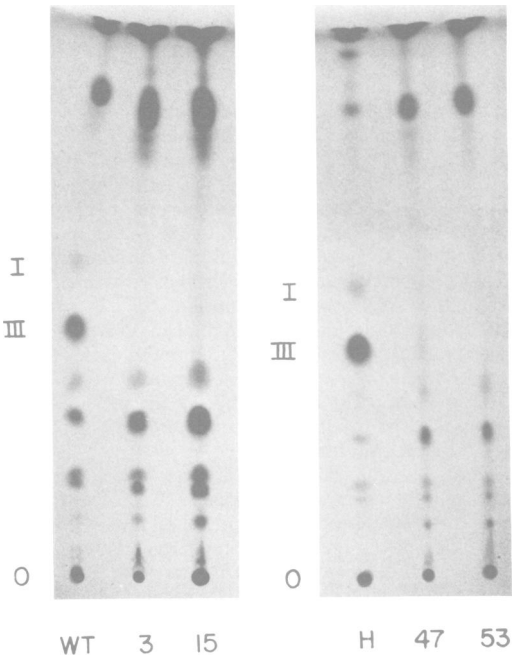

FIG. 1. *Thin-layer chromatographic separation of lipids extracted from filaments (WT) and partially purified heterocysts (H) of wild-type A. variabilis and from filaments of mutants NF-3, 15, 47, and 53. Each spot corresponds to a sample containing* 3×10^7 *heterocysts. O, Origin; I and III, heterocyst envelope glycolipids I and III, respectively.*

DISCUSSION

In order for nitrogenase to function in vivo, the enzyme must be protected against inactivation by oxygen and must be provided with ATP and with a supply of reductant. Our work represents an approach to investigation of the details of the physiological systems which are required to support the activity of the nitrogenase which is present in aerobically grown, heterocyst-forming cyanobacteria.

Both H_2, which is produced by a side reaction during assimilation of N_2, and substrates of the dehydrogenases of the oxidative pentose phosphate cycle can serve as sources of reductant for O_2 or for substrates of nitrogenase within heterocysts (5, 10–12). It has been suggested that the envelope of heterocysts may so extensively restrict the ingress of oxygen that that which enters can be assimilated by the oxidative reactions taking place inside the cells, whereby a very low partial pressure of oxygen is maintained in their interior (16).

Working with intact cells, we have described three experimentally distinguishable classes of mutants which have the potential for nitrogenase activity under anaerobic conditions, but are largely or completely inactivated by oxygen. The oxygen sensitivity of one class, the mutants of which are deficient in the envelope glycolipids of heterocysts, may be accounted for by more rapid penetration of oxygen into their hetero-

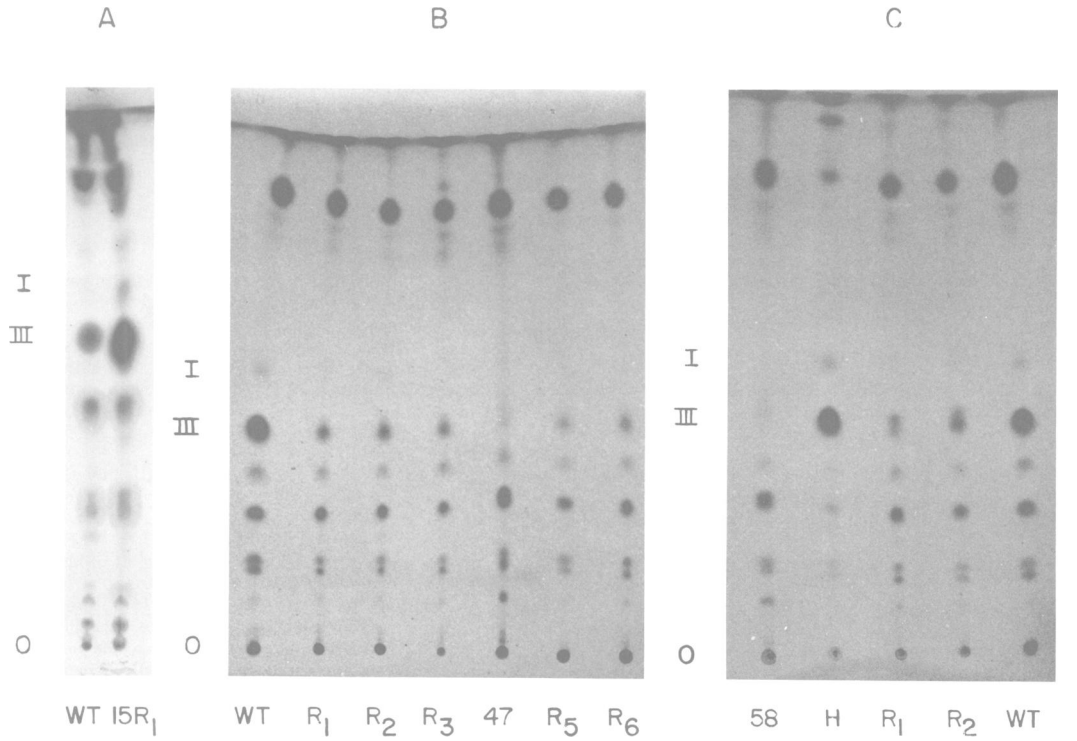

FIG. 2. *Thin-layer chromatographic separation of lipids extracted from filaments (WT) and partially purified heterocysts (H) of wild-type A. variabilis and from filaments of the following mutants and revertants (R_n) of those mutants: (A) NF-15, (B) NF-47, and (C) NF-58. The wild-type and revertant strains were grown with N_2 as nitrogen source; the mutants were grown with nitrate and then transferred to nitrate-free medium. Each sample represents an extract of 3×10^7 heterocysts. O, Origin; I and III, heterocyst envelope glycolipids I and III, respectively.*

cysts than into the heterocysts of wild-type cultures. The fact that it was possible to isolate from a number of these mutants revertants which are capable of aerobic fixation of dinitrogen and which have an increased but still abnormally low complement of heterocyst glycolipids is consistent with the following idea. These glycolipids, which are localized in the envelopes of the heterocysts, may be a part of the oxygen-protective mechanism. However, in the absence of detailed biochemical definition of the lesions in the mutants, it remains possible that the mutations had pleiotropic effects. That is, the effect of the mutations on the system providing oxygen protection may have been different from their effect on the production of the glycolipids of heterocysts.

The mutants of the other two classes have the normal complements of glycolipids, but the mutants of one of those classes require provision of dithionite for the expression of high nitrogenase activity, whereas the one mutant of the other class does not. Specifically, after 4 days of incubation under microaerobic conditions, i.e., exposure to photosynthetically active light under flowing N_2–CO_2, mutants NF-12, 62, and 63 reduce acetylene much more rapidly in the presence of dithionite than in its absence. Their nitrogenase, although relatively inactive, cannot have been irreversibly damaged under microaerobic conditions. Therefore, these mutants must provide some protection against inactivation by oxygen. The fact that they require dithionite for high activity thus implies that, as in a mutant of *Klebsiella pneumoniae* which has been described (15), some component in their pathway of electron transfer to nitrogenase (although not to 2,3,5-triphenyltetrazolium chloride) may be impaired. (In particular, it is possible that the electron-accepting site of their nitrogenase may be modified.) Such a component could be in a pathway separate from those which protect against inactivation by oxygen. Alternatively, the component could be in a pathway common to both processes, but with a limited supply of electrons used preferentially for oxygen protection.

Because glutamine synthetase and/or glutamine may influence the formation of heterocysts (14, 18), we examined the activity of glutamine

synthetase in three mutants in which very high or very low frequencies of heterocysts can be observed (2). Little or no difference was observed in this activity in the mutants relative to wild-type *A. variabilis*.

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