JOURNAL OF BACTERIOLOGY, Mar. 1977, p. 1556-1562

Vol. 129, No. 3
Printed in U.S.A.

Isolation and Preliminary Characterization of Auxotrophs of a Filamentous Cyanobacterium

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Received for publication 21 September 1976

Auxotrophic mutants of the filamentous cyanobacterium *Anabaena variabilis* were isolated by a method in which, after mutagenesis and before penicillin enrichment, mutant and wild-type cells were separated by cavitation. Auxotrophs were identified by their inability to grow on minimal medium, and they were partially characterized by replica plating to media supplemented with single nutrients or specific groups of nutrients. Of the 83 auxotrophs isolated, 65 required an inorganic source of nitrogen for growth. In addition, auxotrophs were isolated that required methionine (six), uracil (two), adenine (one), biotin (two), and nicotinic acid (two). (The number of isolates of each type is indicated in parentheses.) The nutrient requirements of five auxotrophs appeared complex and were not determined. A large proportion of the mutants requiring inorganic fixed nitrogen was altered in the differentiation of heterocysts. The following morphological aberrancies were observed: abnormally high and abnormally low frequencies of heterocysts; thick, uneven heterocyst envelopes; incompletely developed pore regions; very distinct pore regions; and protoplasts separated from the envelope of the heterocyst. Spontaneously occurring, N_2-fixing, prototrophic revertants of mutants with aberrant heterocysts have been isolated at a frequency of 2×10^{-8} to 4×10^{-8} of the cells plated. That most such revertants produced morphologically normal heterocysts is consistent with the idea that heterocysts play an essential role in aerobic N_2 fixation.

Filamentous, heterocyst-forming members of the cyanobacteria (blue-green algae) carry out O_2-evolving photosynthesis and fix atmospheric nitrogen. Their vegetative cells can differentiate into heterocysts and sometimes into spores (akinetes) in a spatially ordered manner. However, application of the powerful techniques of the genetics of procaryotes to the analysis of these physiological and developmental processes is not yet possible in these cyanobacteria because of the lack of highly efficient systems for genetic transfer. A necessary step toward developing such genetic systems is to obtain stable mutants, preferably with biochemically defined lesions resulting from single-point genetic changes.

Drug-resistant and auxotrophic mutants of unicellular cyanobacteria have been described (4, 6, 8, 13) and reviewed (16), and the biochemical bases of some of these lesions have been characterized (6, 8). Morphological variants, e.g., filamentous forms, have also resulted from mutagenesis of unicells (7).

Mutants of filamentous cyanobacteria either resistant to streptomycin or lacking heterocysts and requiring reduced nitrogen for growth have been described (12, 14). Morphological mutants with short trichomes (9) or with variations in the pattern of heterocysts (11, 17) have also been isolated. In none of these mutants has the primary biochemical lesion been identified.

By treatment with a chemical mutagen, followed by procedures involving cavitation to separate cells with mutant and wild-type genomes, penicillin enrichment, and replica plating, we have isolated over 80 auxotrophic mutants of *Anabaena variabilis*. So far, only the nutritional requirements of most of these mutants have been determined. However, further characterization should provide the biochemical definition of the markers that is a prerequisite for a conclusive demonstration of genetic transfer.

MATERIALS AND METHODS

Growth medium. The medium of Allen and Arnon (1) was solidified with 1% agar (Difco Laboratories, Detroit, Mich.) purified by the method of Braun and Wood (2) for plating experiments, and it was diluted eightfold (AA/8) for use with liquid cultures.

Chemicals. All stock solutions were sterilized by filtration through 0.22-μm pore filters (Millipore Corp., Bedford, Mass.). Penicillin G, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)

were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions of NTG-TES (500 μg of NTG per milliliter of 10 mM TES, pH 7) and of penicillin G (5,000 U/ml in distilled water) were made and filter-sterilized immediately prior to use.

Stock solutions of the nutrients (Table 1) were made both individually and in groups at 100 times the final concentration. The solutions were adjusted to pH 7.0 with NaOH or HCl, except that group 6 was adjusted to pH 1.6. Group 3 nutrients were solubilized at pH 11.0, and the pH was then lowered to 7.0.

Alanine, cysteine, isoleucine, leucine, proline, phenylalanine, threonine, tryptophan, tyrosine, valine, uracil, and sodium malate were purchased from Mann Research Laboratory, New York, N.Y.; folic acid, nicotinic acid, calcium pantothenate, thiamine, and sodium pyruvate were purchased from Calbiochem, La Jolla, Calif.; sodium acetate and sodium citrate were obtained from J. T. Baker, Phillipsburg, N.J.; *p*-aminobenzoic acid was purchased from Difco; and riboflavin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. The other nutrients listed in Table 1 were purchased from Sigma. All amino acids with an asymmetric C2 were in the L configuration.

Cell growth and mutagenesis. *A. variabilis* Kütz. (Indiana University culture collection no. 1444 [3]) was grown in 100 ml of AA/8 medium in 500-ml Erlenmeyer flasks at room temperature (ca. 22°C) on a rotary shaker at 100 rpm. Lighting was provided by cool white fluorescent lamps placed approximately 3 feet (ca. 0.9 m) from the cultures, yielding an intensity of approximately 3,200 ergs/cm^2 per s. Liquid cultures were always incubated under these conditions unless indicated otherwise. Under these conditions, the alga grew logarithmically, with a generation time of approximately 24 h. When a culture reached a density of ca. 4×10^6 cells/ml, it was treated in a sonic cleaning bath (model 8845-3, Cole-Parmer Instrument Co., Chicago, Ill.). The Erlenmeyer flask containing the culture was suspended in the water of the bath for 15 min, which allowed cavitation to break cells randomly and yielded filaments averaging 2.5 cells per filament (19). The stock solution of NTG-TES was added to the culture (final concentration, 25 μg of NTG per ml), and the culture was incubated for 6 h. The NTG treatment killed 99.9% of the cells as assayed by plating on minimal medium. This percentage of mortality was close to the optimal determined by Herdman and Carr (4) for the reversion to wild type of a nitrate reductase mutant of *Anacystis nidulans*.

Selection and identification of mutant genotypes. The mutagenized cells were washed free of NTG-TES by two cycles of centrifugation (1,000 × *g* for 10 min) and suspended in AA/8 medium. They were then incubated for 4 days in 10 ml of AA/8 medium supplemented with all nutrients (Table 1). During this incubation period, cell division and, presumably, segregation of mutant genomes took place. The cells were then washed as described above and suspended in 10 ml of the minimal medium AA/8. To separate mutant and wild-type cells physically, we again fragmented the filaments by treatment in the sonic cleaning bath, which yielded single cells and short filaments. Directly after cavitation, the culture was incubated in the light in minimal medium for 2 days to deplete endogenous pools of nutrients in auxotrophic mutants. To enrich for mutant genotypes, the cells were incubated in the light for an additional 2 days with 200 U of penicillin G per ml. The cells were then washed free of penicillin and plated, at 500 to 800 colonies per petri plate, on fully supplemented plates. The plates contained all of the nutrients at full strength, except that the vitamins (group 7) were diluted 10-fold. When the group 7 nutrients were at full strength in fully supplemented plates, the plating efficiency was decreased.

Colonies were either streaked with sterile toothpicks onto petri plates of minimal medium with or without all supplements, or they were replica-plated directly to minimal medium. Colonies that did not

TABLE 1. *Nutrient groups, concentrations of supplements, and composition of diagnostic media*[a]

Nutrient group	Substituents (μM)	Diagnostic media			
		I	II	III	IV
1	Gln (16); Glu, Pro, Arg (80)	+[b]			
2	Lys (3.2); Thr (0.64); Asp, Asn, Met (80)	+	+		
3	Tyr (3.2), Trp (16), Phe (80), His (0.013)	+		+	
4	Cys (1.6); Ser, Gly (16)	+			+
5	Ala (16); Leu (80); Val, Ile (3.2)		+		
6	Adenine (3.2), thymine (0.64), cytosine (80), uracil (16)		+	+	
7	Vitamin B_{12} (0.015); thiamine, *p*-aminobenzoic acid, nicotinic acid, biotin, riboflavin, pyrodoxine·HCl, folic acid, calcium pantothenate (0.8)		+		+
8	Disodium succinate, trisodium citrate, sodium pyruvate, sodium acetate (20); fumaric acid, malic acid (4)			+	
9	NH_4Cl (5,000), TES (10,000)			+	+

[a] Modified from reference (5).

[b] +, Present.

grow or bleached after a few days on minimal medium were transferred from the original fully supplemented medium to form an organized master plate of presumptive auxotrophic mutants on complete medium. The group-level nutritional requirements of the auxotrophs were identified by replica plating with sterile velveteen to the four diagnostic media (Table 1) and to the minimal medium. The nutritional requirements were then defined specifically by replica plating to medium supplemented with individual nutrients.

RESULTS

Auxotrophs were recovered at a frequency of approximately 0.5% of the survivors. Of the presumptive mutants tested on the diagnostic media I through IV, six grew only on the media with group 2 supplements, three grew only with group 6 supplements, four grew only with group 7 supplements, 65 required group 9 supplements, and five (not further studied) appeared to require more than one group of nutrients. By replica plating to individual nutrients within the groups, we identified the following requirements: all group 2 auxotrophs required methionine; of the three group 6 auxotrophs, two required uracil and one required adenine; of the group 7 auxotrophs, two required biotin and two required nicotinic acid. All 65 of the group 9 mutants grew in the presence of 5 mM NH_4Cl and 10 mM TES, pH 7.2, and most but not all grew in the presence of nitrate; when plated on minimal medium, none grew continuously, although most underwent several division cycles, after which the colonies bleached.

Some of the auxotrophs that were unable to grow in the absence of fixed nitrogen produced heterocysts of apparently normal morphology at a frequency similar to that of wild type (cf. Fig. 1G and H), whereas the heterocysts of other mutants were altered in their morphology or development. At least four classes of mutants with morphologically aberrant heterocysts were observed. One class produced heterocysts with envelopes of uneven thickness (Fig. 1A). Another class formed heterocysts whose pore regions were not fully differentiated (Fig. 1B). In the third class, the structures of the pore regions were unusually large and distinct (Fig. 1C). In the fourth class of mutants, the protoplast of the heterocyst appeared to be separated from the cell wall (Fig. 1D) and was sometimes quite shrunken and apparently dead. In addition, mutants with reduced (Fig. 1E) and increased frequencies of heterocysts (Fig. 1F), as compared with the wild type, were found.

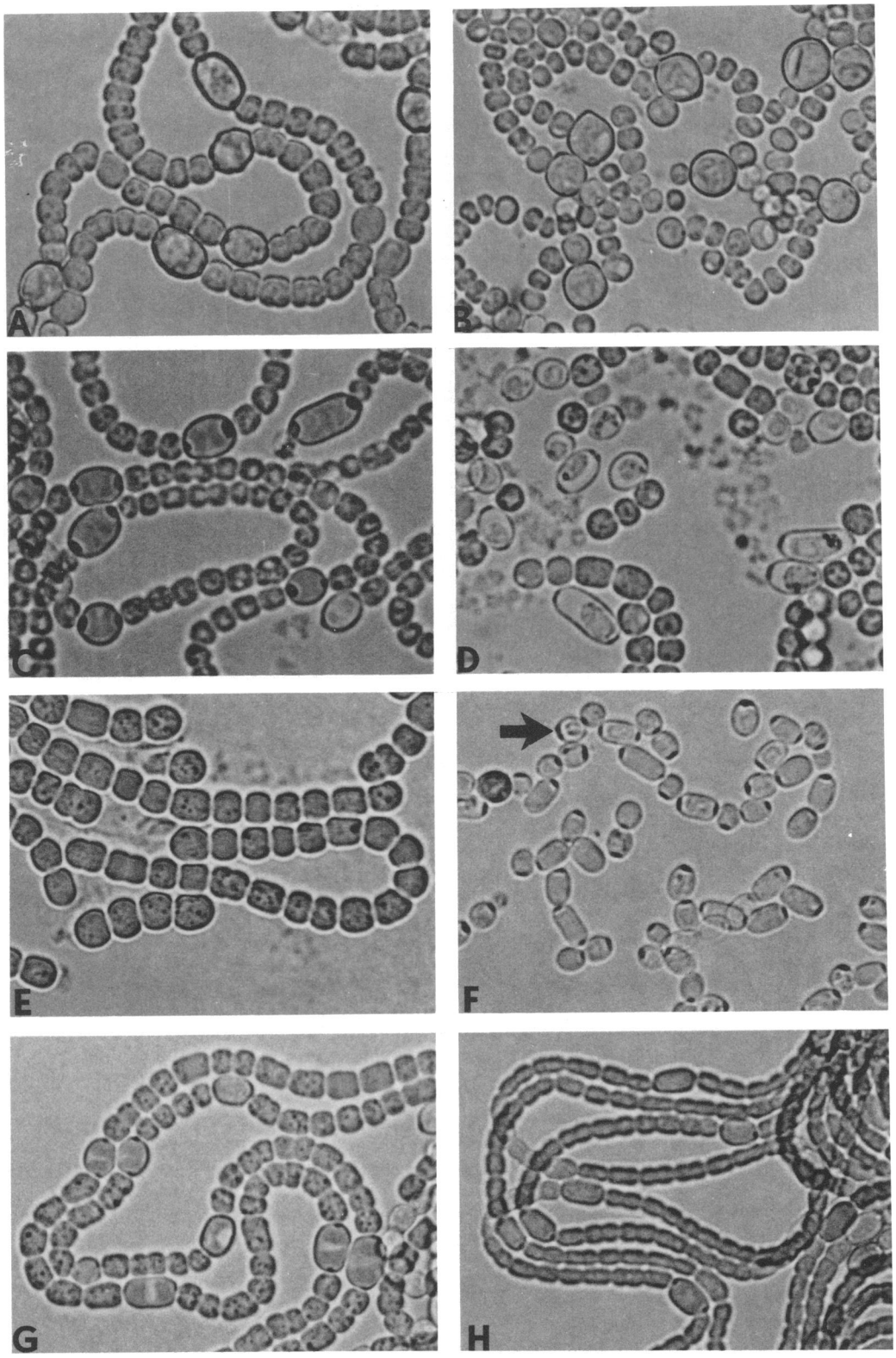
Certain of the morphological mutants exhibited characteristics of different classes. Thus, in some, the heterocysts possessed envelopes of variable thickness and, at the same time, had thickened structures at their poles. In others, heterocysts were produced at an abnormally high frequency (11), and had protoplasts, some of which were separated from the cell envelope (Fig. 1F).

To determine if the observed alterations in heterocyst differentiation were related to the inability of these mutants to grow on nitrogen-free medium, spontaneous revertants capable of fixing N_2 aerobically were obtained by plating a large number of mutant cells on minimal media. Revertants were not isolated for mutants NF16 and NF76 after plating 2×10^8 and 7×10^8 cells, respectively. It may be that the phenotype of these mutants is the result of a lesion that does not easily revert or is not easily suppressed, or the alterations may result from multiple lesions induced by NTG. For the mutants NF30, 14, 15, and 12, two to four revertants appeared as green colonies for 10^8 cells plated on minimal agar. Most of the revertants grew well on minimal agar and produced heterocysts at a frequency similar to that of the wild type (Fig. 2A through D). However, a few of the revertants grew slowly on minimal agar and showed partial reversion of their aberrant morphologies.

DISCUSSION

We have isolated and partially characterized auxotrophic mutants of *A. variabilis*, a filamentous cyanobacterium. After fragmentation of the filaments to one- and few-celled pieces by cavitation, we could use genetic techniques that are standard for work with unicells, namely, chemical mutagenesis, penicillin enrichment, and replica plating. Close to four-

FIG. 1. *Wild-type A. variabilis and mutants unable to grow with N_2 as nitrogen source. Auxotrophs were isolated as described in the text, replica plated to minimal medium, and allowed to grow for 8 days. A cover slip was then placed over the cells on the agar surface, or a sample was taken from the agar surface and squashed under a cover slip on a glass slide. The mutants (A through G) are altered in heterocyst differentiation as indicated: (A) NF30, envelope of uneven thickness; (B) NF16, poorly formed polar structures; (C) NF14, unusually distinct polar structures; (D) NF15, shrunken protoplast; (E) NF76, mutant that rarely differentiated heterocysts; (F) NF12, mutant that has a high frequency of heterocysts. Some heterocysts have shrunken protoplasts (arrow); (G) NF24, unable to grow with N_2 as nitrogen source but heterocysts of normal appearance and frequency; (H) wild-type A. variabilis.* ×1,020.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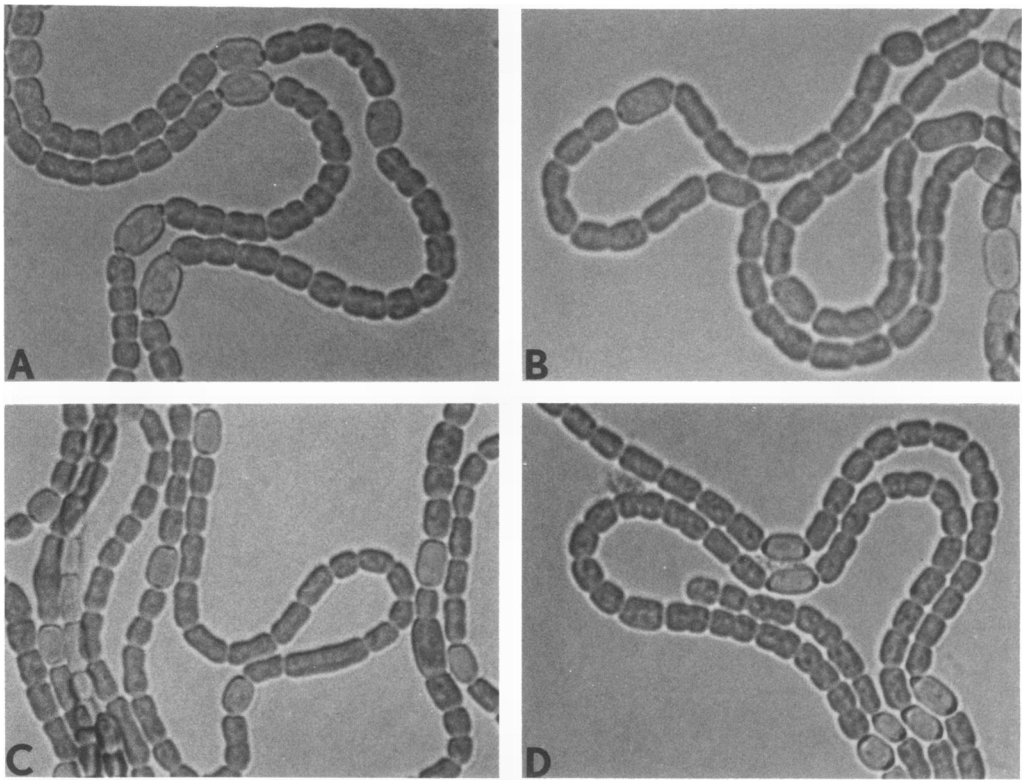

FIG. 2. *Revertants of mutants unable to grow with N_2 as nitrogen source. Revertants of the mutants shown in Fig. 1 were isolated by plating 10^7 to 10^8 mutant cells per plate on petri plates of minimal medium and picking green colonies that arose. Revertants were transferred to minimal medium and observed after 8 days of growth as described in Fig. 1. Revertants of mutants (A) NF30, (B) NF14, (C) NF15, and (D) NF12 are shown. ×1,020.*

fifths of the mutants isolated required ammonium (or nitrate) for growth. A few such mutants have also been isolated from other filamentous cyanobacteria (12, 14). We have in addition, and for the first time, isolated mutants from a filamentous cyanobacterium that have specific organic requirements, viz., methionine, uracil, adenine, nicotinic acid, and biotin. Currently, we are biochemically characterizing the specific abnormalities of various auxotrophs that we have isolated.

By using the complete medium described in this paper, it should, in principle, be possible to obtain auxotrophs requiring any individual supplement. However, the number of metabolically different mutants isolated was relatively small. One reason for this small count may be that some of the nutrients were present in the fully supplemented medium at concentrations too low to support growth. This interpretation is supported by the observation that one of the methionine-requiring mutants was able to grow on 100 μM cysteine, but not on medium IV that contained only 1.6 μM cysteine.

The low concentrations of nutrients used were established as follows: in preliminary experiments, approximate maximal concentrations of the individual nutrients were found that, when included in the medium of a master plate, permitted quantitative replica plating. When combined at these concentrations, the nutrients inhibited growth. Therefore, the concentrations of all amino acids, purines, and pyrimidines were reduced by a factor of 25, and vitamins by a factor of 250, so that growth would not be impaired. Organic acids were present at 1/25th of the concentrations used by Herdman et al. (5). For the isolation of auxotrophs with specific requirements other than those that we obtained, it may be necessary to grow the mutagenized cells in a medium containing a higher concentration of the specific supplement. We know of no reason to doubt that many more types of mutants can be isolated.

Track autoradiography after fixation of ^{13}N-labeled nitrogen gas has shown that heterocysts are major sites of nitrogen fixation (18), a conclusion that has been confirmed with isolated heterocysts (10, 15, 15a). Much circumstantial evidence has been interpreted as indicating that heterocysts are the sole sites of nitrogen fixation in heterocyst-bearing cyanobacteria under aerobic conditions. This evidence is equally consistent with, but does not prove rigorously, even the weaker interpretation that heterocysts are essential for, although not necessarily the sole sites of, aerobic N_2 fixation in these cyanobacteria.

Most of the mutants we have isolated that do not fix N_2 have either structurally aberrant heterocysts or have altered heterocyst frequencies. However, some mutants requiring a source of fixed nitrogen possess heterocysts that appear structurally normal in the light microscope. At a higher level of resolution, the heterocysts from these mutants might prove to be structurally aberrant. Alternatively, these mutants might be changed in some essential component of the nitrogen-fixing system (possibly nitrogenase itself), loss of which does not affect the morphology of the heterocysts.

Aerobic N_2 fixation, if dependent upon the presence of heterocysts, would be inhibited by a regulatory mutation that greatly reduces the frequency of heterocyst formation. N_2 fixation might be impaired in a regulatory mutant in which the great majority of vegetative cells differentiate to form heterocysts, because provision to the heterocysts, from vegetative cells, of products of photosynthesis required for N_2 fixation would be curtailed or stopped. Mutations affecting the envelope or membranes of heterocysts might prevent N_2 assimilation by impairing a system that protects nitrogenase against inactivation by oxygen or provides it with a reductant or adenosine 5'-triphosphate.

If the altered heterocyst differentiation of certain mutant strains is directly related to the inability of those strains to grow on N_2, then revertants that are able to grow on N_2 as well as the wild type does, should differentiate "normal-looking" heterocysts at frequencies similar to that of the wild type. However, it is not expected that all revertants will be identical to the wild type, inasmuch as it is also possible that a mutant will regain the ability to fix N_2 by suppression of the mutation rather than actual correction at the exact site of the mutation. In fact, we observed that some of the revertants of the N_2-nonfixing strains grew significantly more slowly and were lighter green on minimal agar than the wild type. These revertants were still somewhat altered in heterocyst differentiation, although in all cases the alteration was less severe than in the original mutant. Other revertants were macroscopically indistinguishable from the wild type on minimal agar and differentiated "normal-looking" heterocysts at frequencies similar to wild type. Although suggestive, these reversion studies do not prove that the observed heterocyst aberrancies are directly related to the inability of the mutants to fix N_2, because it is possible that the mutations affecting heterocyst differentiation have independent, pleiotropic effects, one of which relates to heterocyst morphology or frequency, whereas another relates to nitrogen fixation. A direct relationship between heterocysts and the total fixation of N_2 by filaments would be demonstrated if it could be shown that the mutation affected the structure of some specific activator or repressor of heterocyst differentiation or some enzyme, lipid, or polysaccharide specific for heterocysts, and that the mutation had no other direct effect.

ACKNOWLEDGMENTS

This work was supported by the U.S. Energy Research and Development Administration under contract E(11-1)-1338 and by Public Health Service grant CA-18133-01 from the National Cancer Institute.

ADDENDUM IN PROOF

Recently, we have isolated revertants of NF76. Heterocysts of normal appearance were present in these revertants.

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