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A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium

(*Anabaena* / *nif*)

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ABSTRACT In many filamentous cyanobacteria nitrogen fixation occurs in differentiated cells called heterocysts. Filamentous strains that do not form heterocysts may fix nitrogen in vegetative cells, primarily under anaerobic conditions. We describe here two functional Mo-dependent nitrogenases in a single organism, the cyanobacterium *Anabaena variabilis*. Using a *lacZ* reporter with a fluorescent β -galactoside substrate for *in situ* localization of gene expression, we have shown that the two clusters of *nif* genes are expressed independently. One nitrogenase functions only in heterocysts under either aerobic or anaerobic growth conditions, whereas the second nitrogenase functions only under anaerobic conditions in vegetative cells and heterocysts. Differences between the two *nif* clusters suggest that the nitrogenase that is expressed in heterocysts is developmentally regulated while the other is regulated by environmental factors.

Filamentous cyanobacteria of the genus *Anabaena* serve as a simple prokaryotic model for developmental control of gene expression. When deprived of a source of fixed nitrogen, about every 10th photosynthetic vegetative cell in the cyanobacterial filament differentiates into a morphologically and physiologically distinct cell called a heterocyst (1, 2). The primary function of heterocysts is nitrogen fixation, the reduction of atmospheric dinitrogen to ammonia mediated by the enzyme nitrogenase. Nitrogenase is very oxygen labile; hence, nitrogen fixation is restricted to anaerobic environments. Heterocysts provide the requisite anaerobic environment because their cell envelope limits oxygen entry and they lack oxygen-evolving photosystem II, which is characteristic of vegetative cells (2). Within a filament heterocysts differentiate in a semiregular pattern, thus providing spatial separation of nitrogen fixation from oxygenic photosynthesis in what is functionally a one-dimensional multicellular organism (3).

Among nitrogen-fixing cyanobacteria that do not differentiate heterocysts there does not appear to be a single mechanism for protection of nitrogenase from oxygen and different strains show a range in oxygen tolerance (4, 5). In many nonheterocystous cyanobacteria, photosynthesis is temporally separated from nitrogen fixation, which occurs only at night (6–8). For other nonheterocystous cyanobacteria that fix nitrogen aerobically in the light without apparently differentiated cells, little is known of the mechanisms for protecting nitrogenase from oxygen (9, 10); however, nitrogenase activity in laboratory-grown cultures is significantly enhanced by lower oxygen tensions (4). Thus, low oxygen tensions are probably necessary for optimal nitrogenase activity.

The heterocystous cyanobacterium, *Anabaena* sp. strain PCC 7120 (hereafter, *Anabaena* PCC 7120), has a large cluster of *nif* genes (including *nifBSUHDKEN*) that encode a Mo-dependent nitrogenase system (11). The *nifB*-*fdxN*-*nifS*-*nifU*

operon is interrupted by a 55-kb insertion in *fdxN* and the *nifD* gene has an 11-kb insertion, both of which are excised during heterocyst differentiation (12–14). The 11-kb element is prevalent in heterocystous cyanobacteria (15) but is missing in all nonheterocystous cyanobacteria examined to date (4). The *nif* genes of *Anabaena variabilis* ATCC 29413 homologous to those of *Anabaena* PCC 7120 have been cloned and partially mapped (16); they contain the 11-kb excision element, but not the 55-kb excision element (17). In addition to that *nifHDK* cluster, a different putative *nifHD* segment, transcribed within hours after the onset of nitrogen starvation under anaerobic conditions, was cloned from *A. variabilis* (18, 19); however, no other *nif* genes were identified in that second *nif* cluster.

Using Southern hybridization, we found evidence for two copies of genes in *A. variabilis* that hybridized to *nifB* and *nifD* probes from *Anabaena* PCC 7120, whereas only one copy of those genes was evident in the latter strain. These genes were not part of the vanadium-dependent *vnf* system encoding nitrogenase 2 that we previously cloned (20). We demonstrate here that there are two large clusters of *nif* genes in *A. variabilis*. One functions under aerobic or anaerobic growth conditions exclusively in heterocysts, while the other *nif* cluster functions only under anaerobic growth conditions in vegetative cells and in heterocysts.

MATERIALS AND METHODS

Strains and Growth Conditions. *A. variabilis* FD is a derivative of *A. variabilis* ATCC 29413 that can grow at 40°C and can support the growth of bacteriophages better than the parent strain (21). *A. variabilis* FD and strains derived from that strain were grown photoautotrophically in liquid cultures in an 8-fold dilution of the medium of Allen and Arnon (22) (AA/8) as described (20). Cyanobacterial cultures were maintained on AA or on BG-11 (23) medium solidified with 1.5% Difco Bacto agar (24). When appropriate, antibiotics were added to plates at the following concentrations: neomycin (Nm), 40 μ g/ml; ampicillin (Ap), 20 μ g/ml; chloramphenicol (Cm), 25 μ g/ml; erythromycin (Em), 5 μ g/ml. In liquid cultures antibiotic concentrations were as follows: Nm, 5 μ g/ml; Em, 5 μ g/ml.

Escherichia coli strains JM109 and HB101 containing plasmids were grown overnight in L broth or on L agar plates (10.0 g of NaCl per liter, 10.0 g of tryptone per liter, 5.0 g of yeast extract per liter, and, for plates, 1.5% Bacto agar, Difco) at 37°C. When appropriate, antibiotics were added at the following concentrations: kanamycin or Ap, 50 μ g/ml; tetracycline, 12 μ g/ml; Cm, 25 μ g/ml.

Southern Hybridizations. Genomic DNA was extracted from cyanobacteria by dispersing cells in a vortex with glass

beads in the presence of phenol (25). Radioactive probes, prepared by the random primer extension technique (26), were hybridized to filters at 58°C in 6× SSC/5× Denhardt's solution/0.5% SDS.

Cloning of *nif2* Genes and Construction of *lacZ* Fusion Strains. The *nif2* genes were identified as weakly hybridizing plaques during a screening of a λ EMBL3 genomic library of *A. variabilis* with probes from the *nifHD* and *nifK* genes of *Anabaena* PCC 7120 (kindly provided by R. Haselkorn, University of Chicago). Preliminary mapping of DNA from several of these plaques provided evidence that they were not the well-characterized *nif* genes previously reported for *A. variabilis* but might contain a putative *nifHD* region reported earlier (18). This latter region, kindly provided to us by R. Hirschberg (University of Missouri, Kansas City) on plasmid pAN101, allowed us to confirm that the weakly hybridizing *nif* genes from our library contained the *nifHD* region she had reported. A variety of probes containing the *nif* genes of *Anabaena* PCC 7120 or the *nif1* genes of *A. variabilis* (kindly provided by J. Golden, Texas A&M University, College Station, TX) were used to map the two clusters.

The *nifBS2* region (where an *fdxN* homolog might have been found) and the *nifEN* regions were sequenced on both strands. The fusion of the *nifEN2* genes has been confirmed by sequencing the junction region from two independent genomic clones. For sequencing reactions we used fluorescent dideoxy-terminators with the Applied Biosystems automated sequencing system.

Plasmid pJE35 was constructed as follows. A 2.8-kb *Hind*III fragment containing the *nifHD2* genes was first cloned into pUC118 and then cleaved at the *Cla*I site in *nifD2*. The *E. coli lacZ* gene (27) (without a promoter) followed by the neomycin/kanamycin (Nm/Km) resistance cassette C.K3 (28) with blunt ends was inserted in the *Cla*I site (after the ends were made blunt) as shown in Fig. 1. The *Hind*III fragment with the *lacZ*-Nm^R insert was cloned into the mobilizable vector pRL271 (29) to produce pJE35, which was transferred to *A. variabilis* strain FD by conjugation (20, 30). Plasmid pTT216 was constructed by cloning a 3.5-kb *EcoRV* fragment containing the *nifHD1* region into the *Sac*I site of pARO180 (a mobilizable version of pUC18) (31) and then replacing the *Kpn*I fragment (from within *nifD1* to the polylinker of pARO180) with the *lacZ*-Nm^R insert (with *Kpn*I ends) such that the *nifHD1* promoter drives *lacZ*. This plasmid was transferred to

strain FD by conjugation to produce strain TT216. Recombinant cyanobacterial strains JE35 and TT216 with the modified *nif* genes integrated into the chromosome by single recombination were identified by their antibiotic resistance and verified by Southern analysis of chromosomal DNA using appropriate *nif* gene probes.

Acetylene Reduction and β-Galactosidase Assays. Cells were grown aerobically in the light with shaking in medium AA/8 with 5.0 mM fructose, 5.0 mM NH₄Cl, and 10 mM Tes (pH 7.2). Exponentially growing cells were washed with AA/8 and resuspended in AA/8 with 5.0 mM fructose and incubated aerobically or anaerobically in the same medium. Anaerobic cultures contained 10 μM dichlorophenylmethylurea (to inhibit oxygen evolution from photosystem II) in serum-stoppered flasks flushed thoroughly with argon. One-milliliter samples were removed for acetylene reduction (32) or for β-galactosidase assays (33).

In Situ Localization of β-Galactosidase Activity. Cells grown aerobically or anaerobically were fixed in 0.01% glutaraldehyde at 25°C for 15 min and washed with water. Cell pellets in minimal volume were resuspended in 15 μl of 100 μM 5-decanoylamino fluorescein di-β-D-galactopyranoside (C₁₂-FDG) (Molecular Probes) in 25% dimethyl sulfoxide (modified from ref. 34). Cells were incubated in the dark at 37°C until fluorescence was microscopically visible (15–60 min). Filaments were washed, resuspended in one drop of Vectashield (Vector Laboratories), an antibleaching agent, and photographed with a fluorescein filter set (excitation, 450–490 nm; dichroic, 510 nm; barrier, 520 nm) on a Zeiss upright microscope, with or without a 560-nm shortpass filter. Exposure times for photographs shown here were 1–8 sec (Kodak Gold Ultra 400 film), depending on the objective. Cells that did not express β-galactosidase required exposure times of 30–60 sec to produce very dim green images with the 560-nm filter.

RESULTS

Cloning of *nif2* Genes of *A. variabilis*. During our studies of the *vnf* genes of *A. variabilis* (20) we found evidence by Southern hybridization for two copies of genes that hybridized to *nifB* and to *nifD* probes from *Anabaena* sp. strain PCC 7120, whereas only one copy of these genes was evident in the latter strain. A genomic library yielded two different clones: one had restriction sites identical to those of the *A. variabilis* *nif* genes that are homologous to the *Anabaena* PCC 7120 *nif* genes (16, 17), which we call *nif1*. A second clone, containing the cluster we call *nif2*, had restriction sites similar to the *nifHD* region described by Hirschberg *et al.* (18). Mapping and partial sequencing of these two clusters (data not shown) have confirmed that these two *nif* clusters are different (Fig. 1) and that the *nif2* cluster contains the *nifHD* region described by Hirschberg *et al.* (18). Three features of the *nif2* cluster are strikingly different from *nif1*: (i) there is no 11-kb insertion in the *nifD* gene; (ii) the region between *nifB2* and *nifS2*, which has been

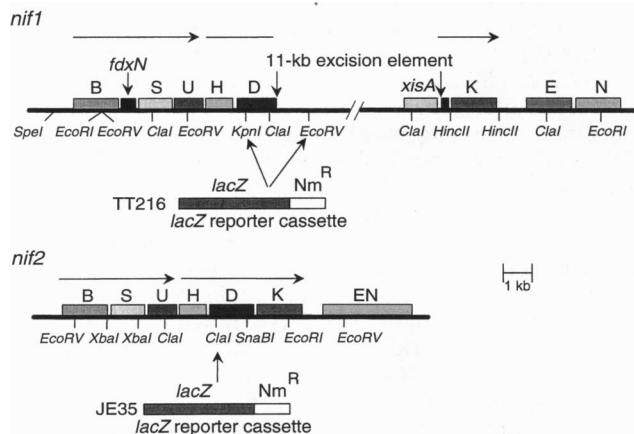


FIG. 1. Maps of *nif1* and *nif2* gene clusters from *A. variabilis*. Positions of genes were determined by Southern hybridization of restriction fragments to *nif* genes of *Anabaena* PCC 7120 and by partial sequencing. The labels "TT216" and "JE35" indicate the genotype of the fusion strains that resulted from the insertion of the *lacZ*-Nm^R cassette in the *nif1* and *nif2* clusters at the restriction sites shown. Arrows indicate transcripts determined by Northern analysis (data not shown).

<i>nifEN2</i>	1	PFLDINQERHHPYAGYVGMVEMARELDEALYSPVWGQVRKSALWQEGVGV
<i>nifEN1</i>	1	PFLDINQERHHPYAGYVGMVEMARELYEALYSPVWGQVRKSALWQEGVGV
<i>nifEN2</i>	51	QSRSGAEQGRKTVVQNSH-----KSAVNPLKQSQPLGAALAF
<i>nifEN1</i>	51	LAHEYTSNHDHILASIEELI*MAIVTLPNKSVAVNPLKQSQALGASLAF
<i>nifEN2</i>	101	GLKGVMLPHFGSQGCTAFKVMVLVRHFREAIPLSTTAMTEVTTILGGEDN
<i>nifEN1</i>	101	GLKGMIPPLPHFGSQGCTAFKVVVLVRHFREAIPLATTAMTEVTTILGGEDN

FIG. 2. Comparison of the deduced amino acid sequences of the region at the junctions of *nifEN*. There are 29 more nucleotides in this region of *nifEN1* than in *nifEN2*. Double dots indicate similar amino acids; single dots indicate unrelated amino acids. The termination site for *nifE1* and the initiation site for *nifN1* are underlined (they overlap by one nucleotide). The gap shown for *nifEN2* is placed arbitrarily.

Table 1. Hybridization of DNA from cyanobacterial strains to *nif2* genes of *A. variabilis*

Strain	Symbiosis	Heterotroph	<i>nif2</i> *	Source
<i>A. variabilis</i> ATCC 29413	None	+	+	C. P. Wolk
<i>Anabaena</i> sp. PCC 7120	None	—	—	C. P. Wolk
<i>Anabaena</i> sp. PCC 7118	None	—	—	C. P. Wolk
<i>Nostoc</i> sp. PCC 7121	None	—	—	C. P. Wolk
<i>Anabaena</i> sp. strain M131	None	—	—	C. P. Wolk
<i>Nostoc</i> sp. ATCC 29150	None	+	—	C. P. Wolk
<i>Anabaena</i> sp. strain V5	<i>Azolla</i> †	+	+	S. Shestakov
<i>Anabaena</i> sp. strain FSR	<i>Azolla</i> †	+	+	W. J. Zimmerman
<i>Anabaena</i> sp. strain PNB	<i>Azolla</i> †	+	+	W. J. Zimmerman
<i>Anabaena</i> sp. strain 9RC	<i>Azolla</i> †	+	+	W. J. Zimmerman
<i>Anabaena</i> sp. strain ARAD	<i>Azolla</i> †	+	+	W. J. Zimmerman
<i>Nostoc</i> sp. strain INDIA	<i>Azolla</i> †	+	—	W. J. Zimmerman
<i>Nostoc</i> sp. strain XNB	<i>Azolla</i> †	+	—	W. J. Zimmerman
<i>Nostoc</i> sp. strain 2RC	<i>Azolla</i> †	+	—	W. J. Zimmerman
<i>Nostoc</i> sp. strain Mac	<i>Macrozamia</i>	+	—	J. C. Meeks

*Hybridization of chromosomal DNA from cyanobacterial strains to *nifHD2* or *nifK2* genes of *A. variabilis* ATCC 29413.

†Free-living cyanobacterial isolate cultured from *Azolla*.

sequenced completely, shows no similarity to the *fdxN* gene; and (iii) the *nifEN2* genes are fused into a single open reading frame (Fig. 2). Partial sequences of both *nif* clusters (>3 kb) show about 75% sequence identity within coding regions. In contrast, segments of *nifI* and the homologous regions in *Anabaena* PCC 7120 show about 95% sequence identity (17, 32).

Prevalence of the *nif2* Genes in Other Cyanobacteria. Using the *nifHD2* genes as a probe we screened chromosomal DNA digests of several heterocystous cyanobacteria for *nif2* homologues. Only a few cyanobacterial strains, all isolated from the water fern *Azolla* that forms a symbiotic association with some cyanobacteria (35), had strong hybridizing bands and those bands were very similar in size to those of *A. variabilis* (Table 1). Those same strains also have the genes for the V-dependent nitrogenase (20) and, thus, all appear to be very closely related, if not identical, strains (32, 35).

Nitrogenase Activity of *nifI* and *nif2* Systems. In *A. variabilis*, nitrogenase activity, as measured by acetylene reduction, appeared under aerobic conditions about 12 hr after the removal of fixed nitrogen; however, nitrogenase activity was detected within 2 hr after nitrogen starvation under anaerobic conditions (Fig. 3) (19). The appearance of nitrogenase in aerobic cultures at about 12 hr corresponded to the time of heterocyst differentiation. Expression of the *nif* genes in

Anabaena sp. PCC 7120 is restricted to heterocysts and nitrogenase is not made until after heterocysts differentiate, even under anaerobic conditions (27). The expression of nitrogenase in *A. variabilis* under anaerobic conditions long before heterocysts differentiated suggested that the expression and regulation of nitrogenase genes are different in this strain.

Transcription of the *nifI* and *nif2* Clusters. To measure transcription of the *nifI* versus *nif2* gene clusters, we constructed strain JE35, in which a promoterless *lacZ* reporter gene was placed in the chromosome under the control of the *nifH2* promoter, and strain TT216, in which *lacZ* was under the control of the *nifH1* promoter. The sites of insertion of the *lacZ*-Nm^R cassette in these two gene clusters are shown in Fig. 1. β -Galactosidase activity was detected under anaerobic conditions in strain JE35 within 1 hr after removal of fixed nitrogen from the medium, but no activity was detected under aerobic conditions (Fig. 4A). In strain TT216, β -galactosidase activity increased at about 12 hr, when heterocysts differentiated, under either aerobic or anaerobic conditions (Fig. 4B). Thus, in *A. variabilis*, expression of the *nif2* cluster required anaerobic conditions and the genes in this cluster were transcribed before heterocysts were formed; however, the question of whether the *nif2* genes were expressed in all vegetative cells or only in cells destined to become heterocysts could not be answered by these assays. In addition, we wanted to determine whether the *nif2* genes were expressed in heterocysts under anaerobic conditions, since they were not expressed even after heterocysts differentiated under aerobic conditions (Fig. 4A).

In Situ Localization of *nifI* and *nif2* Expression. Expression of the *lacZ* reporter was visualized by fluorescence microscopy in filaments of strains JE35 and TT216 incubated with the substrate C₁₂-FDG. Aerobically grown cells of TT216 produced large amounts of fluorescein only in heterocysts; vegetative cells were a faint to moderate red because of fluorescence of the biliproteins that are present in vegetative cells but diminished in heterocysts (Fig. 5A). An additional shortpass filter blocked the red fluorescence of the biliproteins, rendering the vegetative cells essentially invisible while heterocyst fluorescence remained strong (Fig. 5B), thus confirming that *nifI* expression was restricted to heterocysts. Anaerobically grown cultures of TT216 also had bright green fluorescent heterocysts and red fluorescent vegetative cells (Fig. 5C). The same filaments viewed through the 560-nm filter showed no evidence of expression of *nifI* genes in vegetative cells under anaerobic conditions (Fig. 5D). In strain JE35 (*nif2::lacZ* fusion) there was no visible expression of *nif2* genes in either vegetative cells or heterocysts of aerobically grown cells (data not shown). Strain JE35 expressed the *nif2* genes under

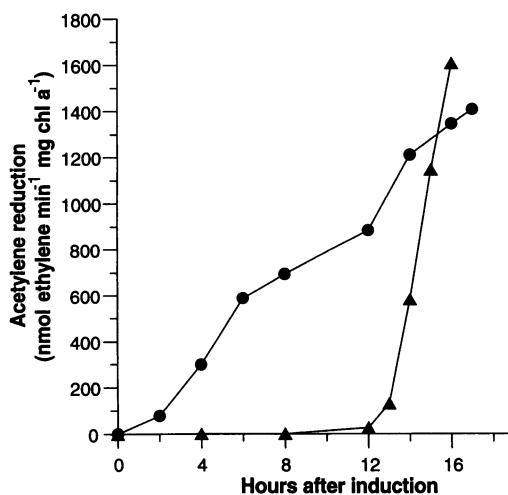


FIG. 3. Acetylene reduction by wild-type strain FD. Cells grown with fixed nitrogen were washed free of fixed nitrogen at 0 time for induction of nitrogenase aerobically (▲) or anaerobically (●).

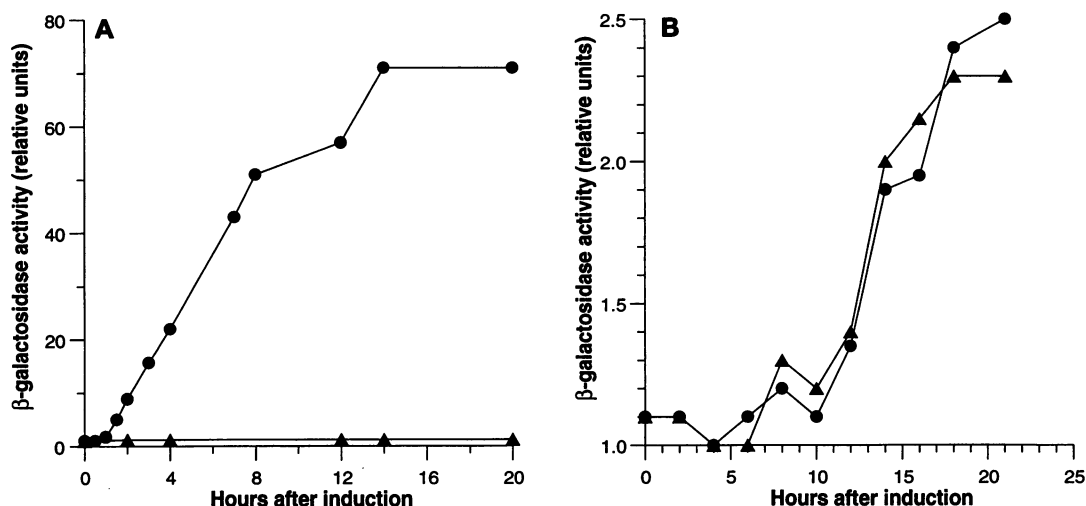


FIG. 4. Expression of *nif1* and *nif2*. (A and B) Strain JE35 (*nif2::lacZ* fusion) (A) or strain TT216 (*nif1::lacZ* fusion) (B) was grown and induced as described in the legend to Fig. 3. β -Galactosidase activity, normalized to cell density, is expressed as a ratio relative to the basal activity (defined as 1) measured in nitrate-grown cultures: for JE35 the basal activity was 10 Miller units (33); for TT216 it was 19 Miller units. ▲ and ●, Aerobically (▲) or anaerobically (●) grown cultures.

anaerobic conditions within 6 hr after nitrogen deprivation (Fig. 5E). The *nif2* genes were expressed in vegetative cells and in heterocysts that formed under anaerobic conditions in strain JE35 (Fig. 5F). Vegetative cells of strain JE35 grown anaerobically after induction retained biliproteins; thus, the photographs of strain JE35 shown here were taken with a filter to block the red. Aerobically grown filaments of JE35 that had

been induced to form heterocysts prior to incubation under anaerobic conditions also expressed the *nif2* genes in vegetative cells and in heterocysts (data not shown).

DISCUSSION

The nitrogenase of the heterocystous cyanobacterium *Anabaena* PCC 7120 functions exclusively in heterocysts (27)

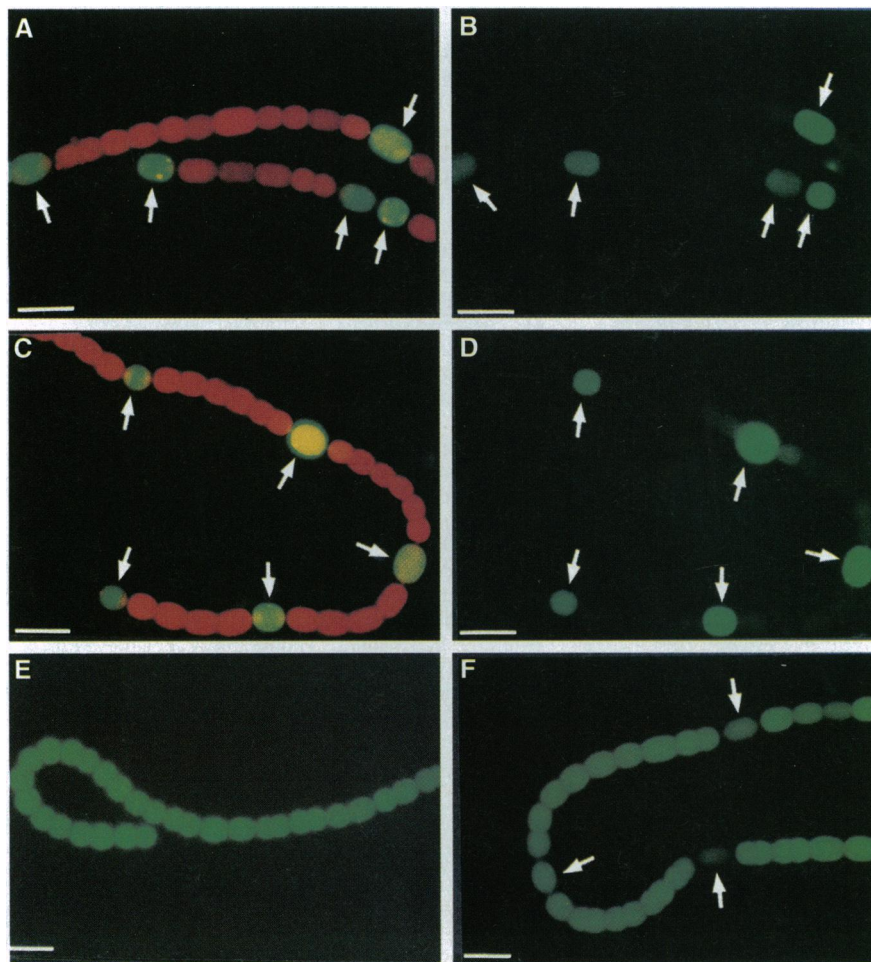


FIG. 5. *In situ* expression of *nif1* and *nif2*. (A and B) Strain TT216 (*nif1::lacZ* fusion) was induced aerobically for 24 hr as described in the legend to Fig. 3 prior to incubation with C_{12} -FDG (34). A and B differ only in that filaments in B were photographed with a 560-nm shortpass filter to block the red fluorescence of the biliproteins. (C and D) Strain TT216 was induced anaerobically for 24 hr as described in the legend to Fig. 3; same filament without (C) or with (D) a 560-nm filter. (E and F) Strain JE35 (*nif2::lacZ* fusion) was induced anaerobically for 6 hr (E) or 24 hr (F) prior to incubation with C_{12} -FDG. Both photographs were taken with a 560-nm filter. White arrows indicate heterocysts identified from light micrographs. (Bars = 10 μ m.)

as does the homologous *nif1* system of *A. variabilis* (Fig. 5 A–D). The V-dependent nitrogenase of *A. variabilis* functions under aerobic conditions and requires the *nifB1* gene (32); therefore, the *vnf*-encoded nitrogenase probably also functions in heterocysts. We describe here a type of alternative nitrogenase in *A. variabilis*, encoded by the *nif2* genes, that functions in all cells, but only under anaerobic conditions.

The similarity in overall organization of the *nif1* and *nif2* clusters suggests either that both of these sets of genes evolved from a common ancestor or that they arose by gene duplication in this strain. Differences such as lack of the *fdxN* gene and the 11-kb excision element, the fusion of the *nifEN2* genes into a single open reading frame, and the relatively low degree of similarity for the regions thus far sequenced suggest that these clusters did not diverge within this strain. It is more likely that they diverged from an ancient common ancestor and that the *nif2* system has been acquired more recently by *A. variabilis*, possibly by gene transfer from a nonheterocystous cyanobacterium. Little is known about the organization of *nif* genes in nonheterocystous cyanobacteria; however, like the *nif2* genes, they lack the 11-kb excision element (15) and the genes function primarily under anaerobic or microaerobic conditions in vegetative cells (4, 5).

The expression of the *nif1* genes exclusively in heterocysts under aerobic and anaerobic conditions indicates that this cluster is regulated by developmental factors as are the *nif* genes in *Anabaena* PCC 7120 (27). In contrast, the *nif2* genes, like those of most nitrogen-fixing prokaryotes, including nonheterocystous cyanobacteria, appear to be regulated directly by environmental factors such as the availability of fixed nitrogen and oxygen tension. The apparent transcription rate of the *nif2* cluster was much greater than that of *nif1*, as indicated by the accumulation of large amounts of β -galactosidase in strain JE35 (Fig. 4). This may result from the expression of the *nif2* genes in vegetative cells that greatly outnumber heterocysts. It is also possible that β -galactosidase is not completely released from the thick-walled heterocysts by permeabilization and, thus, the assay may not accurately reflect the enzyme activity *in vivo* for these cells.

The *nif1* nitrogenase activity per heterocyst was substantially greater than the *nif2* nitrogenase activity on a per cell basis; however, activity on a per filament basis was similar for the two systems (Fig. 3). Thus, it would appear that either system could support diazotrophic growth under the appropriate conditions. However, expression of the *nif2* nitrogenase under anaerobic conditions did not prevent the differentiation of heterocysts and expression of the *nif1* system. Thus, for this organism having two functional Mo-dependent nitrogenases, the *nif1* system appears to dominate, even under anaerobic conditions. The *nif2* system, although fully functional, appears to serve as an auxiliary system that does not suppress expression of the *nif1* system. Such a supplemental system could be advantageous for cells that are starved for fixed nitrogen under anaerobic or microaerobic conditions. These conditions are encountered in algal mats, particularly at night when no oxygen is produced from photosynthesis. The absence of the *nif2* genes in many common laboratory strains of heterocystous cyanobacteria further supports a supplemental role for this system in nitrogen fixation. However, the conservation of these fully functional genes also suggests that they provide a selective advantage to *A. variabilis*.

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