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# Characterization of Genes for a Second Mo-Dependent Nitrogenase in the Cyanobacterium *Anabaena variabilis*

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***Anabaena variabilis*** **ATCC 29413 is a filamentous heterocystous cyanobacterium that fixes nitrogen under a variety of environmental conditions. Under aerobic growth conditions, nitrogen fixation depends upon differentiation of heterocysts and expression of either a Mo-dependent nitrogenase or a V-dependent nitrogenase in those specialized cells. Under anaerobic conditions, a second Mo-dependent nitrogenase gene cluster, *nifII*, was expressed in vegetative cells long before heterocysts formed. A strain carrying a mutant gene in the *nifII* cluster did not fix nitrogen under anaerobic conditions until after heterocysts differentiated. The *nifII* cluster was similar in organization to the *nifI* cluster that is expressed in heterocysts and that includes *nifBSUHDKENXW* as well as three open reading frames that are conserved in both cyanobacterial *nif* clusters.**

In *Anabaena* spp., aerobic nitrogen fixation occurs in terminally differentiated cells called heterocysts that form in a semiregular pattern in a filament in response to nitrogen starvation. These spatially segregated cells differ from vegetative cells both structurally and biochemically (reviewed in references 6 and 39). Heterocysts lack photosystem II activity; hence, they do not evolve oxygen (30), and additional envelope layers that are present in the heterocyst probably function in the protection of nitrogenase from oxygen (29). Under aerobic conditions nitrogenase genes are expressed exclusively in heterocysts of *Anabaena* spp. (7, 38). In many nonheterocystous cyanobacteria, photosynthesis is temporally separated from nitrogen fixation, which occurs only at night (11, 26, 33). 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 (10, 12).

*Anabaena variabilis* ATCC 29413 has three sets of nitrogenase genes: the *nifI* and *nifII* clusters, which encode Mo-nitrogenases, and the *vnf* cluster, which encodes a V-dependent nitrogenase. The *nifI* genes of *A. variabilis* are the homologs of the major *nif* gene cluster of *Anabaena* sp. strain PCC 7120, which includes *nifBSUHDKENXW* (14, 16, 19, 22, 24, 27, 28). In addition, *nifJ*, which is not part of the major *nif* cluster and is not essential for nitrogen fixation, has been identified (2). The *nifB-fdxN-nifS-nifU* operon in *Anabaena* sp. strain PCC 7120 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 (4, 13, 14). The 11-kb element is prevalent in heterocystous cyanobacteria (23), including *A. variabilis*, but is missing in all nonheterocystous cyanobacteria examined to date (reviewed in reference 8). The *nifI* genes of *A. variabilis* are about 95% identical to the *Anabaena* sp. strain PCC 7120 *nif* genes at the nucleotide level and differ primarily in the absence of a 55-kb excision element in the *fdxN* gene (5, 17, 20). The *nifBI* gene is essential for both the *nifI* nitrogenase and for the *vnf* nitrogenase, but neither *nifSI* nor *nifUI* is required for nitrogen fixation, with or without Mo or V (20).

The V-nitrogenase system of *A. variabilis*, which functions in Mo-starved cells grown with V, is encoded by the *vnfDGKEN* cluster that is similar to that of *Azotobacter vinelandii* (36, 37). These genes are repressed by Mo and function during aerobic growth in some species of heterocystous cyanobacteria, although they are absent in *Anabaena* sp. strain PCC 7120 and most other commonly studied strains.

A second cluster of *nif* genes, transcribed within hours after the onset of nitrogen starvation under anaerobic conditions, has been identified in *A. variabilis* (34, 38). The *nifII* cluster comprises homologs of the *nifI* genes (18, 38), including ferredoxin genes (34). Homologs of the *nifII* genes are not present in *Anabaena* sp. strain PCC 7120 (38). These genes encode a Mo-dependent nitrogenase that functions only under anaerobic conditions. A *lacZ* transcriptional reporter, fused to *nifI* or *nifII* genes in the chromosome, was used to demonstrate, at the single-cell level, that the *nifI* genes are expressed only in heterocysts under aerobic or anaerobic conditions whereas the *nifII* genes are expressed only under anaerobic conditions in vegetative cells and in heterocysts (38).

**Sequence analysis.** The *nifII* gene cluster is strikingly similar in organization to the *nifI*-type clusters of *A. variabilis* and *Anabaena* sp. strain PCC 7120 (Fig. 1). No other cyanobacterial *nif* clusters of this size have been sequenced; however, for those strains for which there are some sequences, *nifU* precedes the *nifHDK* cluster. In the heterocystous strain *Nostoc commune*, the cyanoglobin gene, *glbN*, separates *nifU* and *nifH* (31). There are several major differences between the organizations of the *nif* genes in the two clusters described here. One is the absence of the 11-kb excision element and the *fdxN* gene in *nifII*. The 11-kb excision element is prevalent in heterocystous cyanobacteria (9, 23) but is absent from a heterocystous *Fischerella* sp. (35). That element is not essential for diazotrophic growth in *Anabaena* sp. strain PCC 7120 (4); however, a mutation that prevents excision of the element prevents nitrogenase synthesis (15). In *A. variabilis* there are two ferredoxin genes downstream from *nifWII* that are probably involved in nitrogen fixation (34). In *Anabaena* sp. strain PCC 7120, the *fdxH* gene downstream from *nifW*, rather than the *fdxN* gene, provides ferredoxin for nitrogen fixation (3). An *A. variabilis* strain carrying an *fdxN* mutation fixes nitrogen normally (21). Another difference between *nifI* and *nifII* is the fusion of the

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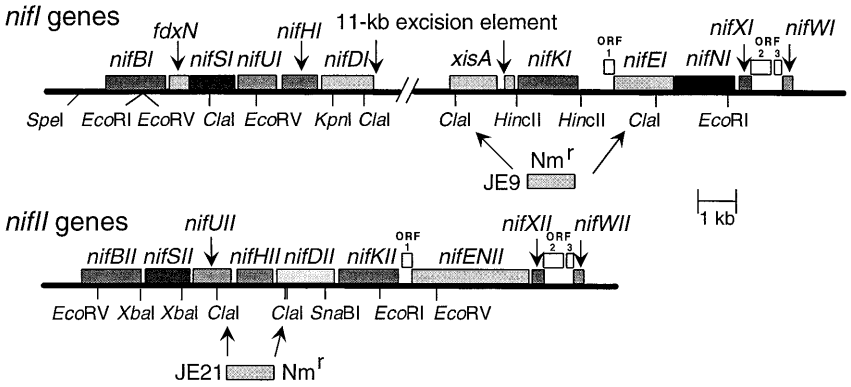


FIG. 1. Maps of the *nifI* and *nifII* gene clusters of *A. variabilis*. The *nifII* genes were identified by their similarity to known *nif* genes. Transcription is from left to right. The *nifDI* gene is interrupted by the 11-kb excision element (4); thus, the carboxy-terminal-coding end of the *nifDI* gene is just upstream of *nifKI*. The arrows indicate sites of insertion of a neomycin resistance cassette to create mutant strains JE9 (in *nifI*) and JE21 (in *nifII*). The *nifII* cluster was sequenced on both strands with single-stranded DNA from restriction fragments located within the cluster. Primers were used to sequence through all restriction sites.

*nifEN* genes into a single open reading frame (ORF) in *nifII* (38). Because *nifE* and *nifN* encode two subunits of a single protein (32), it is likely that the fused protein folds and functions similarly to the protein assembled from two subunits.

The coding regions of genes of the *nifI* cluster that have been sequenced show about 95% nucleotide identity with their homologs in *Anabaena* sp. strain PCC 7120 (5, 20), the only cyanobacterial strain in which all the *nif* genes of this cluster have been sequenced. Therefore, we used the *nif* genes of *Anabaena* sp. strain PCC 7120 (GenBank accession no. U47055) (14, 19, 22, 24, 27, 28) as the basis for sequence comparison for the *nifII* genes. A comparison of these two cyanobacterial *nif* clusters is provided in Table 1. The sequences were 57 to 73% identical at the nucleotide level within coding regions; however, there was no significant similarity between the clusters in the intergenic regions (data not shown). The deduced amino acid sequences for the *nifB*, *nifS*, *nifU*, *nifH*, *nifD*, *nifK*, *nifE*, and *nifN* protein homologs showed 69 to 80% similarity; however, products of three ORFs that are conserved between the two sequences, as well as those of the *nifX* and *nifW* genes, showed only 59 to 65% amino acid similarity. Although the *nifE* and *nifN* homologs were similar, there were 29 more nucleotides in the combined sequences of *nifEI* and *nifNI* than in the fused gene for *nifENII* (38). The termination site for *nifEI* and the initiation site for *nifNI* overlap by one nucleotide.

*Plectonema boryanum* is a filamentous nonheterocystous cyanobacterium that fixes nitrogen only under microaerobic conditions (8, 25). While *Anabaena* sp. strain PCC 7120 and *A. variabilis* fix nitrogen under aerobic conditions in heterocysts using the *nif* or *nifI* genes, respectively, the *nifII* cluster of *A. variabilis* functions, like the *nif* genes of *P. boryanum*, in vegetative cells (38). Alignments of the deduced amino acid sequences for *nifU*, *nifH*, and *nifD* products (data not shown) revealed many positions at which the products of the *nif* genes from *P. boryanum* and the *nifII* genes of *A. variabilis* showed conserved residues that differed from those of the products of the heterocyst-specific *nifI*-type genes. This was particularly striking in the carboxy-terminal region of NifH, which is poorly conserved in all *nifH* gene products (data not shown). The *nifHDII* genes of *A. variabilis* were more closely related to the *nifHD* genes of *P. boryanum*, which lacks heterocysts and fixes nitrogen under anaerobic conditions, than to the *nifHDI*-type

TABLE 1. Comparison of *nif* genes of *Anabaena* sp. strain PCC 7120 with *nifII* genes of *A. variabilis*

| Gene | Sequence comparison | | Deduced protein | | | |
|---|---|---|---|---|---|---|
| | | | Mol wt | | Isoelectric point | |
| | % Nucleotide identity | % Amino acid similarity | PCC 7120 | NifII | PCC 7120 | NifII |
| *nifB* | 73 | 78 | 52,882 | 53,956 | 6.61 | 6.86 |
| *nifS* | 69 | 78 | 43,661 | 42,953 | 6.61 | 6.96 |
| *nifU* | 64 | 71 | 32,135 | 33,031 | 4.61 | 4.88 |
| *nifH* | 73 | 79 | 32,818 | 32,281 | 5.26 | 4.89 |
| *nifD* | 67 | 80 | 55,898 | 55,180 | 7.68 | 6.66 |
| *nifK* | 66 | 69 | 57,581 | 57,650 | 5.46 | 5.86 |
| ORF1 | 65 | 63 | 12,518 | 11,136 | 7.97 | 6.70 |
| *nifE* | 69 | 79 | 52,811 | 98,817[a] | 6.35 | 6.29[a] |
| *nifN* | 63 | 73 | 48,337 | | 4.91 | |
| *nifX* | 64 | 65 | 15,044 | 14,944 | 5.05 | 5.80 |
| ORF2 | 59 | 59 | 17,708 | 17,792 | 7.31 | 8.34 |
| ORF3 | 57 | 61 | 8,228 | 9,805 | 4.80 | 6.60 |
| *nifW* | 62 | 59 | 12,262 | 12,284 | 5.08 | 5.31 |

[a] Deduced product of *nifENII* gene of *A. variabilis*.

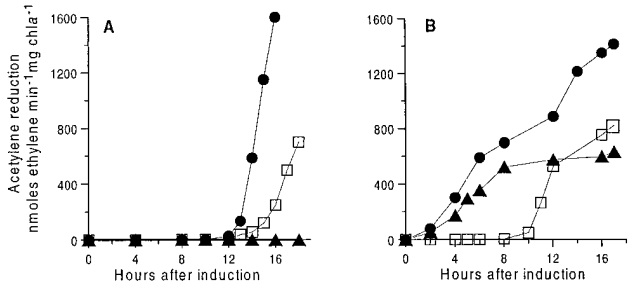


FIG. 2. Acetylene reduction assays of the parent strain, FD (●), and of JE9 (*nifI*) (▲) and JE21 (*nifII*) (□) mutant strains. Cells were grown aerobically in the light with shaking in an eightfold dilution of the medium of Allen and Arnon (AA/8) (1) with 5.0 mM fructose, 5.0 mM $NH_4Cl$, and 10 mM *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.2) to an optical density at 720 nm ($OD_{720}$) of about 0.7. Cells were washed with AA/8, resuspended in 50 ml of AA/8 with 5.0 mM fructose to an $OD_{720}$ of <0.05, and incubated in the same medium. Anaerobic cultures contained 10 μM DCMU (to inhibit oxygen evolution from photosystem II) in serum-stoppered flasks flushed thoroughly with argon. Experiments were repeated at least three times, and representative graphs are provided for aerobic (A) and anaerobic (B) cultures.

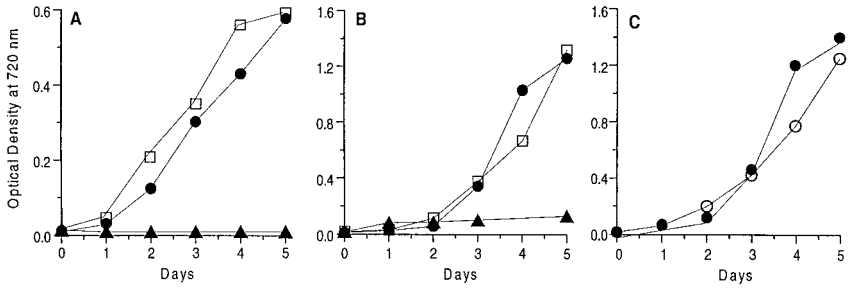


FIG. 3. Growth of the parent strain, FD (●), and of JE9 (*nifI*) (▲), JE21 (*nifII*) (□), and JE994 (○) mutant strains under nitrogen-fixing conditions. Cells were grown and washed as described in the legend for Fig. 2 except that anaerobic cultures were flushed thoroughly with dinitrogen instead of argon. Experiments were repeated at least three times, and representative graphs are provided for aerobic (A) and anaerobic (B and C) cultures.

genes of *Anabaena* sp. strain PCC 7120 and *Fischerella* sp. Therefore, although the *nifI* and *nifII* clusters of *A. variabilis* reside in the same strain, they are phylogenetically distinct.

**Mutant characterization.** Mutant strains JE9, with a $Nm^r$ gene replacing the *xisA-nifEII* region of the *nifI* genes, and JE21, with a $Nm^r$ gene replacing the *nifUII-nifDII* region of the *nifII* genes (Fig. 1), were constructed by replacing the wild-type allele in the chromosome with the mutant copy. This was accomplished by the transfer of the plasmid containing the altered gene from *Escherichia coli* to *A. variabilis* by conjugation as previously described (20, 36, 37). Nitrogen fixation, as measured by acetylene reduction (20), was not detected under aerobic growth conditions in the parent strain, FD, until 12 to 14 h after the removal of fixed nitrogen from the medium (Fig. 2A), when mature heterocysts were apparent. In mutant JE21 (*nifII* mutant), acetylene reduction was slightly delayed compared to that of the parent strain, but both strains reduced acetylene well (Fig. 2A). In contrast, mutant JE9 (*nifI* mutant) failed to reduce acetylene under aerobic conditions even after heterocysts were observed at about 12 h (Fig. 2A). Under strictly anaerobic conditions, nitrogenase activity was detected about 2 to 4 h after induction in FD and in JE9 (*nifI* mutant), but was not observed in JE21 (*nifII* mutant) until about 11 h after induction, when heterocysts had formed (Fig. 2B). In FD, the specific activity of nitrogenase increased after heterocysts formed; this increase in activity was not observed for JE9, which lacked the NifI nitrogenase (Fig. 2B). There was some variation among experiments in the levels of acetylene reduction, and times of induction varied by 1 to 2 h, especially for JE21; however, the overall timing of expression of the two systems was consistent. In some experiments, the wild-type strain, under anaerobic conditions, reduced acetylene by about 1 h after induction. The early expression of the NifII nitrogenase in wild-type cells did not, however, prevent subsequent differentiation of heterocysts and synthesis of active NifI nitrogenase.

JE9, the *nifI* mutant, did not grow under aerobic conditions in a medium lacking a source of fixed nitrogen (Fig. 3A), even after several weeks of incubation. The strain produced heterocysts as usual about 12 h after deprivation of fixed nitrogen. JE21, the *nifII* mutant, grew at the same rate as the parent strain, FD, under aerobic conditions in a medium lacking fixed nitrogen (Fig. 3A). Thus, the *nifI* genes, but not the *nifII* genes, were essential for diazotrophic growth under aerobic conditions. Under strictly anaerobic conditions strains FD and JE21 (*nifII* mutant) grew well (Fig. 3B). A mutation in the *nifII* genes (JE21) did not affect growth under anaerobic conditions (Fig. 3B) because the *nifI* system, which is expressed in heterocysts under anaerobic conditions (38), was functional.

JE9 grew very slowly under anaerobic conditions (with dichlorophenyl dimethylurea [DCMU] to inhibit oxygen evolution from photosystem II) (Fig. 3B), and did not grow at all under microaerobic conditions (flushed with nitrogen, without DCMU) (data not shown). Therefore, a culture of JE9 was maintained for several weeks under anaerobic growth conditions until good growth was evident, and then those cells were subcultured under anaerobic conditions until a strain, named JE994, that grew much better than JE9 was selected (Fig. 3C). We confirmed that JE994 retained the deletion of the *nifI* genes and that it did not grow under aerobic conditions in the absence of fixed nitrogen (data not shown). Nitrogenase activity under strictly anaerobic conditions was not significantly different in JE994 than in JE9 (data not shown); thus, the nature of the mutation in JE994 is unknown. Despite its ability to fix nitrogen and to grow well diazotrophically under anaerobic conditions, JE994 nevertheless differentiated heterocysts. This suggests that internal sources of fixed nitrogen do not repress heterocyst differentiation. Perhaps only external sources of fixed nitrogen can repress differentiation.

The wild-type strain of *A. variabilis* has been maintained in the laboratory for many years, but never under conditions where the *nifII* genes would function; therefore, it is possible that in truly wild strains the *nifII* system enhances growth, possibly at night in algal mats, when cells may become anaerobic. The maintenance of this large cluster of genes without deletions, insertions, or rearrangements argues that it has been functional in the recent evolutionary past.

**Nucleotide sequence accession numbers.** The GenBank accession number for the nucleotide sequence of the *nifII* gene cluster is U49859, and that for the *nifHI* sequence is U89346.

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