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Characterization of *nifB*, *nifS*, and *nifU* Genes in the Cyanobacterium *Anabaena variabilis*: NifB Is Required for the Vanadium-Dependent Nitrogenase

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Anabaena variabilis **ATCC 29413 is a heterotrophic, nitrogen-fixing cyanobacterium containing both a Mo-dependent nitrogenase encoded by the *nif* genes and V-dependent nitrogenase encoded by the *vnf* genes. The *nifB*, *nifS*, and *nifU* genes of *A. variabilis* were cloned, mapped, and partially sequenced. The *fdxN* gene was between *nifB* and *nifS*. Growth and acetylene reduction assays using wild-type and mutant strains indicated that the *nifB* product (NifB) was required for nitrogen fixation not only by the enzyme encoded by the *nif* genes but also by the enzyme encoded by the *vnf* genes. Neither NifS nor NifU was essential for nitrogen fixation in *A. variabilis*.**

Anabaena spp. are filamentous cyanobacteria capable of fixing dinitrogen in specialized morphologically distinct cells called heterocysts (18, 41). Under diazotrophic growth conditions, vegetative cells compose 90 to 95% of the cells in a filament and contain both photosystems I and II. The other 5 to 10% of cells are heterocysts that have only photosystem I and therefore evolve no oxygen (40). Additional envelope layers in heterocysts may protect nitrogenase from oxygen (38, 39).

Many *nif* genes have been identified in *Anabaena* sp. strain PCC 7120: one large cluster contains *nifB*, *fdxN*, *nifS*, *nifU*, *nifH*, *nifD*, *nifK*, *nifN*, *nifX*, open reading frame 3, *nifW*, open reading frame 1, open reading frame 2, and *fdxH* (8, 23, 30, 33, 36, 37, 42). *nifJ* is not part of the cluster (4). 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 (19, 20). The heterocyst ferredoxin gene of *Anabaena* sp. strain PCC 7120, *fdxH*, is downstream of the other characterized *nif* genes in the cluster (8). The *nif* genes of *A. variabilis* ATCC 29413 that are the homologs of the *nif* genes of *Anabaena* sp. strain PCC 7120 have been cloned and mapped (24), and the 11-kb excision element, but not the 55-kb excision element, has been found in that *nif* cluster (9).

Besides the *nif* genes that encode nitrogenase 1, which requires a Mo cofactor, *Anabaena variabilis* also contains *vnf* genes that encode nitrogenase 2, which requires a V cofactor (53). Nitrogenases that do not require Mo were first described in *Azotobacter vinelandii*, which has nitrogenase 1, nitrogenase 2, and nitrogenase 3. Nitrogenase 3, which requires only Fe in the cofactor, is encoded by the *anf* genes (6, 10, 27). The *vnf* and *anf* systems of *A. vinelandii* do not have homologs of all the genes of the *nif* system. Known *vnf* and *anf* genes include *vnfDGK*, homologous to *nifDK*; *vnfEN*, homologous to *nifEN*; *vnfA*, homologous to *nifA*; and *anfHDGK*, homologous to *nifHDK* (6). The *nifB*, *nifS*, *nifU*, *nifM*, and *nifV* genes are required for the activity of all three nitrogenases of *A. vinelandii* (28, 29). The *vnfEN* genes may also function in the *anf* system (6). The *vnf*- and *anf*-encoded nitrogenases are repressed by Mo and have the ability to reduce acetylene not only to ethylene but also to ethane, which is one criterion for the presence of these alternative nitrogenases (6, 14, 15).

NifB is essential for nitrogenase activity in *Klebsiella pneumoniae* (50), for the *nif*- and *anf*-encoded nitrogenases in *Rhodobacter capsulatus* (48), and for the *anf*-, *vnf*- and *nif*-encoded nitrogenases in *A. vinelandii* (27, 45). NifB-co, made by the NifB protein, has been purified and found to be a small Fe-S cluster that is a precursor of FeMo-co (49). FeMo-co is preassembled and then placed into the apo-MoFe protein (55).

NifS is thought to function by presenting the inorganic sulfide required for the synthesis of the Fe-S clusters contained in both components of nitrogenase (26, 35, 56). NifS was recently purified and shown to be a pyridoxal-phosphate-containing homodimer that catalyzes the specific desulfurization of L-cysteine (56). Therefore, NifS may be the S donor for the formation of the Fe-S clusters in both components of nitrogenase.

NifU may serve a function similar to that of NifS by presenting Fe for the synthesis of the Fe-S clusters of nitrogenase (12). All sequences of *nifU* reveal 8 conserved cysteine residues that are presumed to function in Fe-S cluster assembly (5, 13, 32, 37). NifU is essential for diazotrophic growth in *A. vinelandii* (26) but is not essential in *R. capsulatus* (32). Merrick et al. reported that NifU is essential for diazotrophic growth of *K. pneumoniae* (34); however, studies by Roberts and Brill (43) led these authors to suggest that NifU might not be essential for nitrogen fixation in that strain.

Since the *nifB*, *nifS*, and *nifU* genes are essential not only for nitrogenase 1 but also for nitrogenases 2 and 3 in *A. vinelandii* and since NifB and NifS are required for both the *nif*- and *anf*-encoded nitrogenases in *R. capsulatus*, we investigated the role of these genes in nitrogen fixation in *A. variabilis*. We describe the identification and characterization of *nifB*, *nifS*, and *nifU* in *A. variabilis* and present evidence that NifB is required for both the *nif*- and *vnf*-encoded nitrogenases in this organism.

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 (11). *A. variabilis* FD and mutant strains

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derived from this strain were grown photoautotrophically in liquid cultures in an eightfold dilution of the medium of Allen and Arnon (1) (AA/8) as described previously (53). Cyanobacterial cultures were maintained on AA or on BG-11 (2) medium solidified with 1.5% Difco Bacto Agar (54). When appropriate, antibiotics were added to plates at the following concentrations: neomycin (NM), 40 μg ml^{-1}; spectinomycin (SP), 3 μg ml^{-1}; streptomycin (SM), 3 μg ml^{-1}; ampicillin, 20 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; and erythromycin (EM), 5 μg ml^{-1}. In liquid cultures antibiotic concentrations were as follows: NM, 5 μg ml^{-1}; SP, 0.5 μg ml^{-1}; SM, 0.5 μg ml^{-1}; and EM, 0.5 μg ml^{-1}.

Escherichia coli JM109 and HB101 containing plasmids were grown overnight in L broth or on L agar plates (10.0 g of NaCl $liter^{-1}$, 10.0 g of tryptone $liter^{-1}$, 5.0 g of yeast extract $liter^{-1}$, and for plates, 1.5% Bacto Agar [Difco]) at 37°C. When appropriate, antibiotics were added at the following concentrations: kanamycin (KM), ampicillin, SP, or SM, 50 μg ml^{-1} (except those cultures with the SP-SM antibiotic resistance cassette from pRL58 where SP was used at 100 μg ml^{-1} on plates and SM was used at 25 μg ml^{-1} in liquid); tetracycline, 12 μg ml^{-1}; and chloramphenicol 25 μg ml^{-1}.

Growth experiments. To deplete cells of internal pools of metal ions, wild-type and mutant strains, except strains EL15 and JE9, were grown for at least 15 generations by serial transfer in 50 ml of AA/8 that had been scrubbed free of Mo and V by using activated charcoal (47) as previously described (53). To deplete cells of strain EL15 and strain JE9 of internal Mo reserves, the cultures were first grown in Mo- and V-free AA/8 with 0.5 mM $NaNO_3$ until cells turned yellow and would not become green with repeated additions of nitrate (indicating insufficient Mo for functional nitrate reductase). NH_4Cl (5.0 mM) and 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; pH 7.2) were then added to restore healthy growth. These cultures were washed free of NH_4Cl and TES before use as inocula for Mo- and V-free media for growth experiments and nitrogenase assays. Mo-starved cultures were used to inoculate 50 ml of Mo-free and V-free AA/8 media, all to similar optical densities (720 nm). Mo or V was then added at 1.0 μM to the appropriate flasks. Optical densities were recorded every 24 h for 5 to 8 days. Growth experiments were repeated at least three times, and representative graphs are provided.

Cloning the *nifB*, *nifS*, and *nifU* genes and mutant construction. Plasmid pEL13, containing an interrupted *nifS* gene, was constructed as follows: the 5.0-kb *Spe*I fragment from cosmid 33D12 (24) (kindly provided by C. P. Wolk) which contained the *nifB-fdxN-nifS-nifU* operon was cloned into the *Xba*I site of pUC118, creating plasmid pEL9. pEL9 was digested with *Cla*I, and an NM-KM antibiotic resistance cassette (C.K3) with *Acc*I ends was inserted, creating pEL11. C.K3 is from Tn*5* with a promoter from the *psbA* gene of *Amaranthus hybridus* that confers high-level NM resistance in *Anabaena* sp. strain PCC 7120 (16). A 4.5-kb *Eco*RI-*Sal*I restriction fragment of pEL11, containing the interrupted *nifS* gene, was then subcloned into pRL277 (7) (kindly provided by Y. P. Cai and C. P. Wolk), creating pEL13. pRL277 can be mobilized to *Anabaena* spp. by conjugation but cannot replicate in those hosts, and it carries the Ω SM-SP resistance gene that is expressed well in *Anabaena* sp. strain PCC 7120 (21). For pEL15, containing an interrupted *nifB* gene, the 5.0-kb *Spe*I fragment from cosmid 33D12 was ligated into pUC118, creating pEL10 (the opposite orientation of pEL9). pEL10 was digested with *Eco*RV, which interrupts *nifB*, and C.K3, with blunt ends, was inserted to give pEL14. pEL14 was digested with *Ssp*I, and the 3.2-kb fragment was inserted into the *Eco*RV site of pRL277, creating pEL15. pEL17, containing an interrupted *nifU* gene, was constructed with pEL1, a plasmid with a 4.1-kb *Cla*I fragment from cosmid 33D12 containing *nifU* (kindly provided by C. P. Wolk) and ligated into pUC118. pEL1 was digested with *Eco*RV, and the 1.96-kb SM-SP resistance cassette, obtained from pRL58 (3) (kindly provided by C. P. Wolk) was inserted, interrupting *nifU* and creating pEL16. A 6.0-kb *Bam*HI restriction fragment from pEL16 was inserted into pBR322 at the *Bam*HI site, creating pEL17. pJE9, containing a deletion of part of the 11-kb excision element, part of *nifD*, and all of *nifK*, was constructed by cloning a 7.5-kb *Eco*RI fragment from pAM434 (9) (kindly provided by James Golden) into pUC118, creating pJE2. A 4.4-kb *Cla*I fragment was removed and replaced by C.K3, creating pJE4. A 4.0-kb *Spe*I-*Sac*I fragment from pJE4 was cloned into pRL271 (similar to pRL277, but carrying chloramphenicol and EM resistance [7]), yielding pJE9, which lacks the end of *nifD* and all of *nifK*.

Plasmids containing mutated *nifB*, *nifS*, *nifU*, and *nifDK* genes of *A. variabilis* in vectors that can be mobilized by the broad-host-range conjugative plasmid RP4 (i.e., pRL271, pRL277, or pBR322) were conjugated to *A. variabilis* FD as described previously (53). Antibiotic-resistant exconjugant colonies of *A. variabilis* were visible after 6 to 8 days. Colonies were patched onto two BG-11 plates: one with the antibiotic for which the mutated cyanobacterial gene carried resistance and the other with the antibiotic for which the vector portion of the cargo plasmid carried resistance. Colonies that exhibited resistance to the former antibiotic and sensitivity to the latter were possible double recombinants. Putative double-recombinant exconjugant colonies were streaked on BG-11 agar with the appropriate antibiotic to ensure that they were axenic and then subjected to three rounds of growth and sonication as described previously (53). Chromosomal DNA was isolated from the putative recombinant cells, and Southern hybridization, using the appropriate cyanobacterial gene as a probe, was used to verify that the antibiotic resistance gene was inserted in the cyanobacterial gene of interest and to demonstrate complete segregation of wild-type and mutant alleles.

Complementation of the *nifB* mutation. A 2.7-kb *Hin*dIII-*Sal*I fragment containing the region upstream of *nifB* and the coding region of *nifB* to the *Hin*dIII site that is 59 bp from the termination signal for *nifB* was removed from pEL10 (see section above for construction of pEL10) and inserted into the *Hin*dIII-*Sal*I sites of plasmid pBluescript KS+ (Stratagene), creating plasmid pTT227. The 3′ end of *nifB* was added to pTT227 as a 180-bp fragment created by PCR using primers designed to amplify the region from the *Hin*dIII site near the end of *nifB* to the start of *fdxN*. This PCR-generated fragment contained at one end the natural *Hin*dIII site in *nifB* and at the other end a *Bam*HI site introduced in the primer at the start of the *fdxN* gene; thus, the PCR-generated fragment was cloned into pTT227 as a *Hin*dIII-*Bam*HI fragment, creating plasmid pTT230. The region of pTT230 containing the PCR-generated fragment was sequenced from the *Bam*HI site through the *Hin*dIII site to confirm that no mutations had been introduced by PCR. pTT230 was not mobilizable by conjugation; therefore, a cassette containing the *bom* site and EM and chloramphenicol resistance genes from pRL1075 (kindly provided by C. P. Wolk) was excised as a 5.0-kb *Bgl*II fragment and inserted at the *Bam*HI site of pTT230, creating pTT232.

Plasmid pTT232 was mobilized by conjugation to the *nifB* mutant strain EL15 as described above, selecting for Nm^r Em^r colonies. One such colony was designated EL15-232 and used for further analysis. The insertion of an additional copy of *nifB* upstream of the inactivated copy (see Fig. 1B) in EL15-232 was confirmed by Southern hybridization with a *nifB* probe using chromosomal DNA isolated from cells grown either with or without added nitrate in the presence of NM and EM (data not shown). There was no difference in restriction pattern of the two cultures, thus confirming that growth of EL15-232 in the absence of a source of fixed nitrogen did not induce rearrangement of the genes to restore an intact *nifB-fdxN-nifS-nifU* operon.

RNA isolation and analysis. RNA was extracted from cells harvested from 1.0 liter of culture by vortexing the cells with glass beads in the presence of phenol and chloroform and then pelleting the RNA through a 5.7 M CsCl cushion by ultracentrifugation to remove the DNA (22). Northern (RNA) analysis was performed according to the method of Sambrook et al. (46) except that the running buffer contained formaldehyde at a final concentration of 0.22 M and 1.5 μl of ethidium bromide (1.0 mg ml^{-1}) was added directly to RNA samples before loading. This produced less diffuse RNA bands and eliminated the need for destaining the gel after running. Approximately 20 μg of RNA in a volume of 10 μl was loaded per lane. Filters were hybridized to radioactive probes prepared by the random primer extension technique (17) as previously described (53).

Acetylene reduction assays. Cultures were concentrated to an optical density of approximately 0.8 (720 nm) and added to 10-ml serum bottles in 1.0-ml aliquots. The bottles were sealed with gas-tight serum stoppers, injected with 1.0 ml of acetylene gas, and placed in an illuminated 30°C shaking water bath for 30 min. Then 1.0-ml samples of gas were removed via a hypodermic needle and injected into a Shimazu gas chromatograph equipped with a 6-ft (ca. 2-m) Poropak N column. The column temperature was 55°C. The ethylene or ethane peak areas were compared with ethylene and ethane standards. Chlorophyll *a* determinations for each 1.0-ml aliquot were made from methanol extracts according to the method of MacKinney (31).

RESULTS

Cloning the *nifB*, *nifS*, and *nifU* genes of *A. variabilis*. The *nifB* gene of *Anabaena* sp. strain PCC 7120 was used as a probe of restriction digests of chromosomal DNA from *A. variabilis* ATCC 29413 and of cosmid 33D12, which contains the *nif* region of *A. variabilis* (9, 24), to identify the approximate location of *nifB* (data not shown).

Restriction fragments from upstream of *nifH* in cosmid 33D12 were subcloned, partially sequenced on one strand, compared with the sequence of the *nifB-fdxN-nifS-nifU* genes of *Anabaena* sp. strain PCC 7120 (37), and found to contain virtually identical genes. The *fdxN* gene was found between *nifB* and *nifS* as it is in *Anabaena* sp. strain PCC 7120 (36) (Fig. 1). Sequence data from one strand, representing about 3 kb of *nifB-fdxN-nifS-nifU*, including the promoter region, showed these genes to be 95% identical at the nucleotide level to *nifB-fdxN-nifS-nifU* of *Anabaena* sp. strain PCC 7120 (36, 37) (data not shown).

Construction of *nifB*, *nifS*, *nifU*, and *nifDK* mutants. Figure 1A indicates the restriction sites in *nifB*, *nifS*, and *nifU* that were interrupted by antibiotic resistance genes to construct mutant strains EL15, EL13, and EL17, respectively. Similarly, the *nifB*-complemented strain, EL15-232, was constructed by the addition of a wild-type copy of *nifB* upstream of the mutated copy by single recombination between a wild-type copy of

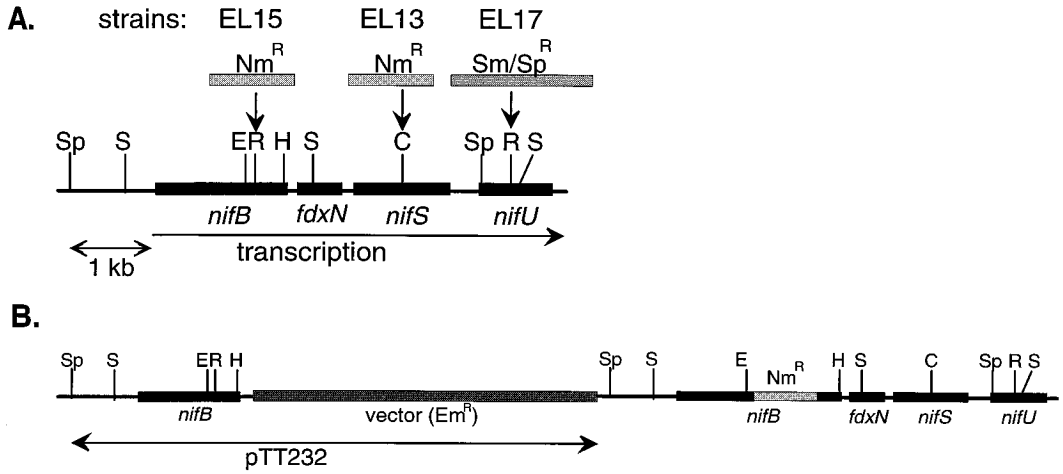

FIG. 1. (A) Restriction map of *nifB*, *fdxN*, *nifS*, and *nifU* in *A. variabilis*. Arrows from the antibiotic resistance genes point to the restriction sites in the *nif* genes where the antibiotic resistance gene was introduced. Insertions of either the Nm^r Nm cassette or the Sm^r-Sp^r cassette followed by recombination of the mutated gene into the chromosome via double-reciprocal recombination yielded strains EL15 (*nifB*), EL13 (*nifS*), and EL17 (*nifU*). C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; R, *Eco*RV; S, *Ssp*I; Sp, *Spe*I. (B) Organization of *nifB* genes in strain EL15-232 in which the insertion mutation of EL15 was complemented by addition to the chromosome of a wild-type copy of the *nifB* gene in plasmid pTT232.

nifB on plasmid pTT232 and the mutated copy in the chromosome of EL15. The organization of EL15-232 in the *nifB* region of the chromosome is shown in Fig. 1B.

Mutant characterization. The *nifB*, *nifS*, and *nifU* mutant strains were grown diazotrophically, and their growth was compared with the growth of the parent strain, *A. variabilis* FD (Fig. 2). Strain EL13 (*nifS* mutant) and strain EL17 (*nifU* mutant) grew nearly as well as the parent strain, lagging only slightly. Strain EL15 (*nifB* mutant) failed to grow in the absence of a source of fixed nitrogen; however, strain EL15-232, in which the *nifB* mutation was complemented, grew very well diazotrophically (Fig. 2). This suggests that NifB is essential for Mo-dependent nitrogen fixation.

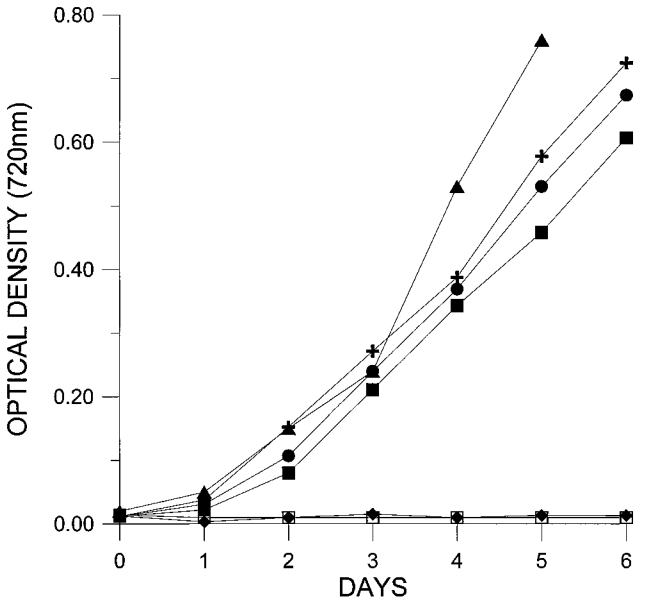

FIG. 2. Diazotrophic growth of parent and mutant strains of *A. variabilis* with Mo. ●, parent strain, FD; +, EL13, the *nifS* mutant; ■, EL17, the *nifU* mutant; ◆, EL15, the *nifB* mutant; □, JE9, the *nifDK* mutant; ▲, EL15-232, the strain in which the *nifB* mutation of EL15 was complemented by a wild-type copy of *nifB*.

In *A. vinelandii* NifB is required by the V-dependent nitrogenase (45). In order to determine whether NifB was similarly required in *A. variabilis*, strain EL15 (*nifB*) was grown diazotrophically with or without Mo or V, and growth was compared with that of parent strain FD and with that of strain JE9 (*nifDK* mutant). Strain JE9, like strain EL15, did not grow under diazotrophic conditions in the absence of Mo and V (Fig. 3A). Unlike strain EL15, however, strain JE9 grew in the absence of Mo when V was present (Fig. 3B). Strain EL15-232, in which the *nifB* mutation was complemented, grew about as well as the parent strain FD in the absence of Mo and grew well with V (Fig. 3). These results suggest that NifB is required by the V-dependent nitrogenase in *A. variabilis.*

Transcription of *nifB*. Total RNA from diazotrophically grown *A. variabilis* FD with or without Mo or V was hybridized to a *nifB* probe (Fig. 4). A transcript was seen in all lanes; thus, *nifB* was transcribed under all these growth conditions. The size of the transcript was similar to that of the major transcript of 1.75 kb seen in *Anabaena* sp. strain PCC 7120 that is probably from *nifB* (37). The transcripts of 3.1 and 4.4 kb that are visible in the Northern blots of RNA from *Anabaena* sp. strain PCC 7120 are present in very small amounts compared with amounts of the 1.75-kb transcript (37) and thus would not be visible on the blot of RNA from *A. variabilis* shown here.

Acetylene reduction assays. The parent strain FD, strain EL13 (*nifS*), and strain EL17 (*nifU*) reduced acetylene to ethylene in the presence or absence of Mo or V (Table 1). When V was present and Mo was absent, all strains reduced ethylene to ethane, a product of the V-dependent nitrogenase. Strain JE9 (*nifDK*) reduced little or no acetylene in the absence of Mo unless V was present. This strain reduced little acetylene in the presence of Mo and V because Mo represses the *vnf* genes (53). Spontaneous pseudorevertants of strain JE9 that grew well in the presence of Mo and V (but failed to grow in the absence of V) reduced acetylene to ethylene and ethane and appear to be Mo transport mutants (52). Data for strain EL15 (*nifB*) are not shown because it did not grow under any of these conditions. Strain EL15-232, in which the *nifB* mutation was complemented, reduced acetylene as well as or better than parent strain FD and produced ethane from cells grown with V (data not shown). The acetylene reduction assay data support

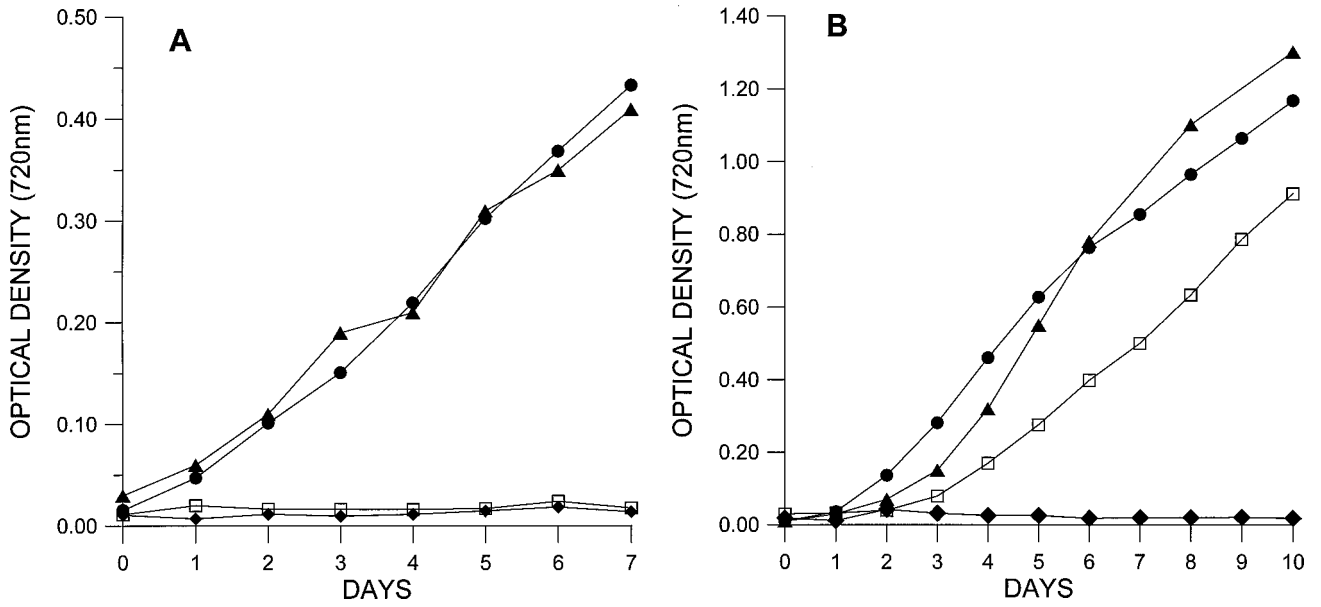

FIG. 3. Diazotrophic growth of parent and mutant strains of *A. variabilis* without Mo. (A) ●, parent strain, FD; ◆, EL15, the *nifB* mutant; □, JE9, the *nifDK* mutant; ▲, EL15-232, the strain in which the *nifB* mutation of EL15 was complemented by a wild-type copy of *nifB*. (B) Same as panel A, but with V.

the conclusion that NifS and NifU are not required by either the Mo-dependent or the V-dependent nitrogenase. In addition, the inability of strain JE9 to grow in the absence of Mo and V and the low acetylene reduction activity of that strain suggest that there is no *anf* (non-Mo, non-V nitrogenase) system in *A. variabilis*.

DISCUSSION

In *A. variabilis nifHDK* and *nifS* have been identified and mapped (9, 24). We report here the characterization of *nifB*, *nifS*, and *nifU* in *A. variabilis*. In order to more accurately map these genes, a total of approximately 3 kb was sequenced on one strand. The sequenced regions showed about 95% identity with the sequence of *Anabaena* sp. strain PCC 7120 (37). There was even greater similarity, 99%, between the sequenced regions of *nif* genes in *A. variabilis* and their homologs in *Anabaena azollae* 1A (25). The latter strain, as well as several other free-living isolates from the fern azolla, hybridized well to a *vnf* gene probe from *A. variabilis* and showed hybridizing restriction fragments similar in sizes to those of *A. variabilis* (53). Thus, *A. variabilis* ATCC 29413 is more closely related to the cyanobacterial isolates from azolla than to *Anabaena* sp. strain PCC 7120.

Strain EL15, the *nifB* mutant, was unable to grow diazotrophically, suggesting that NifB-co, a small Fe-S cluster made by the NifB protein and the precursor of FeMo-co (49), is required for such growth. In *K. pneumoniae* and *R. capsulatus* NifB has also been reported to be essential for diazotrophic growth (48, 50). NifB is required not only by nitrogenase 1 (Mo dependent) but also by nitrogenases 2 (V dependent) and 3 (non-Mo, non-V) in *A. vinelandii* (28). Strain EL15 failed to grow diazotrophically with V in the absence of Mo. In contrast, strain JE9, the *nifDK* mutant, grew under these conditions.

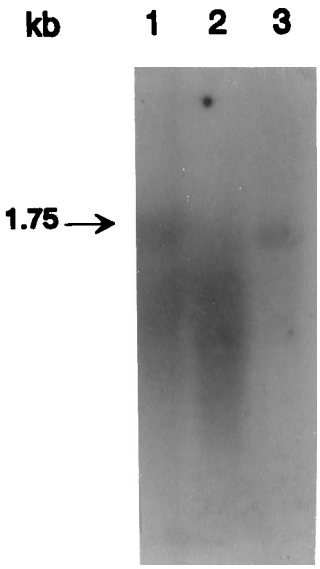

FIG. 4. Northern analysis of *nifB* expression. RNA from cells grown with Mo and without V (lane 1), without Mo and with V (lane 2), or without Mo and without V (lane 3) was hybridized to a 0.7-kb *Eco*RI-*Ssp*I *nifB-fdxN* probe.

TABLE 1. Acetylene reduction by *nif* mutants of *A. variabilis*

Strain	Sp act [nmol of ethylene (nmol of ethane) min^{-1} mg of chlorophyll^{-1} ± SD][a]			
	−Mo		+Mo	
	−V	+V	−V	+V
FD (wild type)	120 ± 27	76 ± 13 (1.3 ± 0.47)	184 ± 42	306 ± 12.5
EL13 (*nifS*)	156 ± 6	178 ± 23 (2.7 ± 0.47)	231 ± 41.7	296 ± 20
EL17 (*nifU*)	84 ± 9.7	188 ± 26 (3.0 ± 0.8)	153 ± 5	241 ± 138
JE9 (*nifDK*)	8.3 ± 1.7	114 ± 29 (2.5 ± 0.5)	ND	3.67 ± 0.9

[a] Mean of three or more determinations. ND, not detected.

This suggests that NifB is required for the V-dependent nitrogenase of *A. variabilis*. Northern analysis showing that *nifB* was transcribed in the absence of Mo supports this hypothesis. Strain EL15-232, in which the *nifB* mutation was complemented without altering the original insertion mutation in *nifB* (Fig. 1B), grew and reduced acetylene with Mo or with V as well as the parent strain; thus, the inability of EL15 to support diazotrophic growth with Mo or with V cannot be attributed to polar effects on downstream genes of the *nifB-fdxN-nifS-nifU* operon.

Diazotrophic growth and acetylene reduction of strain EL13, the *nifS* mutant, were similar to those of the parent strain. There is speculation that NifS and NifU protect the Fe-S clusters from inactivation by O_2 (13). *nifS* mutants of *R. capsulatus* cannot grow diazotrophically (32). *nifS* mutants of *K. pneumoniae* (44) and *A. vinelandii* (26) grow poorly under diazotrophic conditions. In *K. pneumoniae* there is an increased accumulation of inactive Fe protein and, to a lesser extent, of MoFe protein (44). In *A. vinelandii*, a *nifS-nifU* double mutant has nearly no Fe protein activity and a greatly decreased MoFe protein activity compared with strains with either single mutation (26). *nifS* mutants of some species may grow diazotrophically using another protein that may be capable of fulfilling the sulfur-presenting role of NifS. A *nifS*-like gene in *Bacillus subtilis*, a non-nitrogen-fixing organism, has recently been discovered (51). If NifS functions as the sulfur donor for the formation of the Fe-S clusters, perhaps cyanobacteria, which have Fe-S components in the photosynthetic apparatus, have other proteins that can functionally complement the mutation.

The *nifU* mutant, strain EL17, grew and reduced acetylene at a rate similar to that of the parent strain under diazotrophic conditions. This suggests that NifU, whose function may be that of an iron donor for the formation of the Fe-S clusters, is not essential for nitrogen fixation in *A. variabilis*. NifU is not essential for growth in *R. capsulatus* (32) but is essential for growth of *A. vinelandii* (26). NifU may be required for growth of *K. pneumoniae* (34), although another study suggests that NifU is not essential (43).

We have identified a second copy of *nifS* and *nifU* in *A. variabilis*. However, the second copies of these genes are not expressed under aerobic, diazotrophic growth conditions; thus, it is unlikely that they could complement the *nifS* or *nifU* mutants described here (52). Perhaps for both *nifS* and *nifU* there are other gene products in cyanobacteria that serve as S and Fe donors for the Fe-S cluster formation.

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