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Isolation and Characterization of the *vnfEN* Genes of the Cyanobacterium *Anabaena variabilis*

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The filamentous cyanobacterium *Anabaena variabilis* fixes nitrogen in the presence of vanadium (V) and in the absence of molybdenum (Mo), using a V-dependent nitrogenase (V-nitrogenase) encoded by the *vnfDGK* genes. Downstream from these genes are two genes that are similar to the *vnfEN* genes of *Azotobacter vinelandii*. Like the *vnfDGK* genes, the *vnfEN* genes were transcribed in the absence of Mo, whether or not V was present. A mutant with an insertion in the *vnfN* gene lacked V-nitrogenase activity; thus, the *vnfEN* genes were essential for the V-nitrogenase system in *A. variabilis*. Growth and acetylene reduction assays with wild-type and mutant strains suggested that the V-nitrogenase reduced dinitrogen better than acetylene. The similarity of the *vnfEN* genes of *A. variabilis* and *A. vinelandii* was not strong. The *vnfEN* genes of *A. variabilis* showed greater similarity to the *vnfDK* genes just upstream than to the *A. vinelandii vnfEN* genes. Sequence comparisons provide support for the idea that if the *vnf* genes were transferred laterally among bacterial strains, the *vnf* cluster was not transferred intact. It appears likely that the structural genes were transferred before a duplication event led to the evolution of the *vnfEN* genes independently in the two strains. The divergence of the *vnfEN* genes from the *vnfDK* genes suggests that this duplication, and hence the transfer of *vnf* genes, was an ancient event.

Anabaena variabilis ATCC 29413 is a filamentous cyanobacterium that fixes dinitrogen under a variety of environmental conditions. Under aerobic conditions, nitrogen fixation occurs exclusively in cells called heterocysts (18, 27). Heterocysts are terminally differentiated cells that develop in a pattern from certain vegetative cells in the filament in response to limitation in fixed nitrogen (reviewed in reference 44). The primary Mo-dependent nitrogenase (Mo-nitrogenase) in this organism is encoded by a contiguous cluster of *nif1* genes, including *nifB1*, *fdxN*, *nifS1*, *nifU1*, *nifD1*, *nifK1*, *nifE1*, and *nifN1*, that are transcribed only in heterocysts (8, 22, 39). These genes are very similar to the *nif* cluster of *Anabaena* sp. strain PCC 7120 (reviewed in reference 21), which is also transcribed exclusively in heterocysts (16). We have recently characterized an alternative Mo-nitrogenase gene cluster in this strain that is absent in *Anabaena* sp. strain PCC 7120; this second cluster contains genes *nifB2*, *nifS2*, *nifU2*, *nifH2*, *nifD2*, *nifK2*, *nifEN2*, *nifX2*, and *nifW2*, which are transcribed in vegetative cells and in heterocysts only under strictly anaerobic conditions (39). These *nif2* genes are homologs of the *nif1* genes; however, this cluster differs from the *nif1* cluster: there is no *fdxN* gene, nor is there the 11-kb excision element that is present in *nif1* and in the *nif* cluster of *Anabaena* sp. strain PCC 7120. In addition, the *nifE1* and *nifN1* homologs are fused into a single open reading frame, *nifEN2*. Certain *fdx* genes in this organism that are expressed only under anaerobic conditions may also be part of this second system (33).

In the absence of Mo, a V-dependent alternative nitrogenase (V-nitrogenase) functions in *A. variabilis* (36). The structural genes for this nitrogenase are very similar to the *vnfD*, *vnfG*, and *vnfK* genes of *Azotobacter* spp. (5, 24, 29); however, homologs of the *vnfD* and *vnfG* genes of *Azotobacter* spp. are fused into a single open reading frame, *vnfDG*, in *A. variabilis*, while *vnfK* is separate (36). The *vnfD* gene in *Azotobacter vinelandii* encodes the α subunit of V-nitrogenase, *vnfK* encodes the β subunit, and *vnfG* encodes the δ subunit. The δ subunit, which is not present in Mo-nitrogenases, is essential for V-dependent diazotrophic growth in *A. vinelandii* (42). Unlike in *A. vinelandii*, in *A. variabilis* there is no *vnfH* gene, encoding the dinitrogenase reductase of the V-nitrogenase, near *vnfDGK*. A mutant strain of *A. variabilis* containing a deletion of part of *vnfDG* lacks V-nitrogenase activity (36), as does a mutant with an insertion in the *nifB1* gene (25). The *nifS1* and *nifU1* genes are not essential for either the *nif1*-encoded Mo-nitrogenase or the V-nitrogenase in *A. variabilis* (25). The *vnf* genes are not prevalent in bacteria: they have been identified only in some species of *Azotobacter* and in a few strains of cyanobacteria, primarily in strains isolated from the water fern *Azolla* sp. (36).

The Mo-nitrogenase of *A. vinelandii* is a heterodimer containing an α subunit, encoded by *nifD*, and a β subunit, encoded by *nifK*, as well as two identical FeMo cofactors (FeMoco) that are essential for N_2 reduction (reviewed in reference 45). The FeMoco contains homocitrate, Mo, Fe, and S. Synthesis of FeMoco requires *nifB*, *nifH*, *nifV*, *nifQ*, *nifE*, and *nifN* but does not require *nifD* and *nifK* (28, 34, 40). Because NifE and NifN show significant structural similarity with NifD and NifK, respectively, it has been suggested that NifE and NifN serve as a scaffold for FeMoco synthesis (7, 10, 11). NifE and NifN form an $\alpha_2\beta_2$ tetramer that binds NifB-co (30), a FeS precursor to FeMoco (3). A FeV cofactor (FeVaco) has been found in the V-nitrogenase of *A. vinelandii* (35) as well as genes for possible scaffolding proteins for FeVaco, *vnfE* and *vnfN* (43). A *vnfX* gene is immediately downstream of *vnfN*. Unlike NifE and NifN, which are essential for the Mo-nitrogenase, VnfE and VnfN are not essential for V-nitrogenase: NifE and NifN can substitute when the *vnfEN* genes are inactivated (43).

I report here the identification, sequence analysis, and characterization of two genes located directly downstream from *vnfDGK* in *A. variabilis*. These genes are similar to the *vnfE* and *vnfN* genes of *A. vinelandii.*

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli		
HB101	F$^-$ *supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	31
JM109	Δ(*lac proAB*) *recA1 thi supE44 endA1 hsdR17 relA gyrA96* F′ [*traD6 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	31
A. variabilis		
FD	Wild type	9
MB2	*vnfDG*::*npt*	36
TT220	*vnfN*::*npt*	This study
TT220-237	*vnfN*::*npt*/*vnfN*$^+$	This study
Plasmids		
pPE45	pUC118 containing a 6.0-kb *Eco*RI fragment with *vnfKEN*	This study
pRL648	Source of C.K3 cassette encoding Nm^r	15
pRL1075	Source of cassette containing *oriT* site for mobilization by RK2 with Cm^r and Em^r	6
pTT219	*Cla*I-*Bam*HI fragment from pPE45 containing *vnfEN* cloned in pBR322	This study
pTT220	Nm^r gene of pRL648 cloned into unique *Xba*I site of *vnfN* in pTT219	This study
pTT237	pPE45 containing 5.0-kb *Bgl*II fragment from pRL1075 containing *oriT*, Cm^r, Em^r	This study

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 (9). *A. variabilis* FD and mutant strains 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 (36). Cyanobacterial cultures were maintained on AA or on BG-11 (2) medium solidified with 1.5% Difco Bacto agar (38). When appropriate, antibiotics were added to plates at the following concentrations: neomycin, 40 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; and erythromycin, 5 μg ml^{-1}. In liquid cultures, neomycin was used at 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, 10.0 g of tryptone, and 5.0 g of yeast extract per liter and, for plates, 1.5% Bacto-Agar [Difco Laboratories]) at 37°C. When appropriate, antibiotics were added at the following concentrations: kanamycin or ampicillin, 50 μg ml^{-1}, tetracycline, 12 μg ml^{-1}; and chloramphenicol, 25 μg ml^{-1}.

Cloning of the *vnfEN* genes and construction of mutants. The *vnfEN* genes were found on the same λ clone that contained the *vnfDGK* genes (36). Plasmid pTT220 was constructed as follows. pPE45 contained a 6.0-kb *Eco*RI fragment with the *vnfKEN* genes cloned in pUC118. A 5.0-kb *Cla*I-*Eco*RI fragment containing *vnfEN*, removed as a *Cla*I-*Bam*HI fragment from pPE45, was cloned into pBR322 at the same sites, creating pTT219. A Nm^r-Km^r resistance cassette (C.K3) with *Xba*I ends was inserted at the unique *Xba*I site of pTT219 (in *vnfN*), creating pTT220. C.K3 contains the *npt* gene from Tn*5* with a promoter from the *psbA* gene of *Amaranthus hybridus*, which confers high-level Nm^r in *Anabaena* sp. strain PCC 7120 (15). Plasmid pTT237 was constructed by inserting a 5.0-kb *Bgl*II fragment from pRL1075 into the *Bam*HI site of pPE45. pRL1075 contains, within a cassette, the *oriT* site that allows conjugative transfer of plasmids by the broad-host-range plasmid RK2 and Em^r and Cm^r genes (6). Construction of plasmids and strains used in this study is summarized in Table 1. Methods used for gene transfer from *E. coli* to *A. variabilis* as well as selection and screening of cyanobacterial mutants have been described elsewhere (25, 36). Chromosomal DNA was isolated from the putative recombinant cells, and Southern hybridization, using a 2-kb *Hin*dIII-*Eco*RI fragment containing the *vnfN* gene (Fig. 1B), was used to verify correct strain construction and demonstrate complete segregation of wild-type and mutant alleles in TT220.

Southern hybridization and RNA analysis. Genomic DNA and total RNA were extracted from cyanobacteria by vortexing cells with glass beads in the presence of phenol (20). DNA restriction fragments were separated electrophoretically in 0.7% agarose gels by using Tris-acetate-EDTA buffer and were then transferred to nitrocellulose (BA85S; Schleicher & Schuell) (4). Radioactive probes, prepared by the random primer extension technique (17), were hybridized to filters in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.5% sodium dodecyl sulfate at 68°C (4). Northern (RNA) blots were performed as described previously for the *vnfDGK* genes (25), using a 1.3-kb *Hin*dII-*Hin*dIII fragment that spanned *vnfEN* as the probe.

DNA sequence determination. Restriction fragments located downstream from the *Cla*I site near the end of *vnfK* (Fig. 1A) were cloned in pUC118 or pUC119 for sequencing using single-stranded DNA and M13 universal primer (4). Synthetic oligonucleotides were used for sequencing regions lacking a convenient restriction site for subcloning. Sequencing reactions used fluorescent dideoxy terminators with the Applied Biosystems automated sequencing system. Sequence analysis and comparisons with known sequences were performed with MacDNAsis software.

Growth experiments. To deplete cells of internal pools of metal ions, wild-type and mutant strains 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 (32) as previously described (36). To further deplete the growth medium of metals, strain FD was grown in the chemically scrubbed AA/8 medium until the cells became yellow in color and growth ceased, indicating severe nitrogen deficiency and depletion of Mo. The cells were removed from this Mo-depleted medium by filtration through a 0.22-μm-pore-size filter, and this biologically scrubbed sterile medium was used for growth and acetylene reduction assays. Addition of Mo or V to this medium restored normal growth of strain FD, indicating that the biological scrubbing did not deplete the medium of other essential nutrients. Cells of each strain, grown in Mo-depleted medium, were used to inoculate flasks containing 50 ml of Mo-depleted medium, supplemented with either Na_2MoO_4 or V_2O_5 at a final concentration of 1.0 μM each. Optical densities at 720 nm were recorded every 24 h. Growth experiments were repeated at least four times, and representative graphs are provided in Fig. 3.

Acetylene reduction assays. Assays were performed as described previously

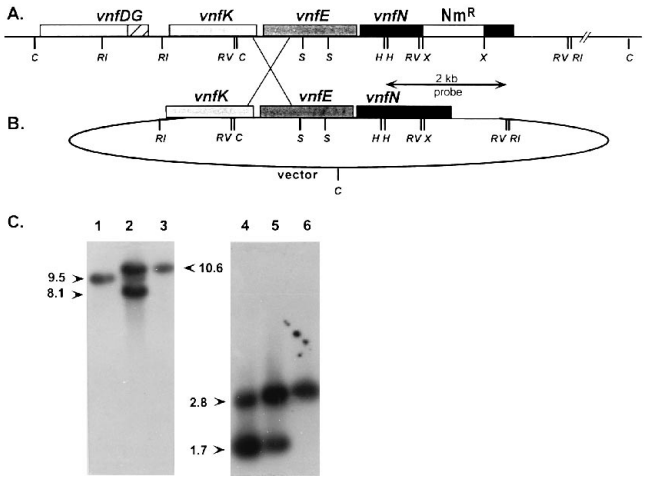


FIG. 1. Map of the *vnf* gene cluster and Southern analysis of mutants. (A) Map of the *vnfDG-vnfK-vnfE-vnfN* cluster of *A. variabilis* showing site of insertion of Nm^r gene in *vnfN* (creating mutant strain TT220). (B) Map of pTT237 containing the wild-type allele of *vnfN* for complementation of mutant strain TT220. The recombinational crossover between the wild-type cluster on the plasmid and the *vnfN* mutation in the chromosome (creating strain TT220-237) is indicated by a large cross joining diagrams A and B. The crossover occurred 5′ to the insertion in *vnfN*; however, the position marked by the cross is arbitrary. *C*, *Cla*I; *RI*, *Eco*RI; *RV*, *Eco*RV; *S*, *Ssp*I; *H*, *Hin*dIII; *X*, *Xba*I. (C) Southern hybridization of DNA from FD (lanes 1 and 4), TT220-237 (lanes 2 and 5), or TT220 (lanes 3 and 6) digested with *Cla*I (lanes 1 to 3) or *Eco*RV (lanes 4 to 6) and hybridized to a 2-kb *Hin*dIII-*Eco*RI probe (see panel B). Sizes are indicated in kilobases.

TABLE 2. Comparisons of deduced amino acid sequences of *vnfEN* genes

Gene comparisons	Amino acid comparison	
	% Identity	% Similarity
vnfE vs. *vnfD*[a]	19.8	56.0
vnfE vs. *vnfE*[b]	16.3	46.0
vnfE vs. *nifE*[c]	19.4	46.5
vnfN vs. *vnfK*[a]	19.2	56.9
vnfN vs. *vnfN*[b]	14.4	52.5
vnfN vs. *nifN*[c]	4.0	13.4

[a] *vnfD* and *vnfK* genes of *A. variabilis* (36).
[b] *vnfE* and *vnfN* genes of *A. vinelandii* (43).
[c] *nifE* and *nifN* genes of *Anabaena* sp. strain PCC 7120 (21a).

(25) except that ethylene and ethane values were normalized to the optical density at 720 nm of the cells rather than to the chlorophyll concentration. This was done because the cultures that grew very poorly (see Fig. 3B and C) had very low amounts of chlorophyll (36) and thus exhibited anomalously high apparent specific activities after normalization to the chlorophyll concentration.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence reported is U51863.

RESULTS

Cloning, sequencing, and sequence comparison of *vnfEN* genes. The *vnfEN* genes were identified by sequencing the region downstream from *vnfDGK* (Fig. 1A). Downstream from *vnfN* in *A. vinelandii* is *vnfX* (43), and *nifX* follows *nifN* in *Anabaena* sp. PCC 7120 (21a); however, in *A. variabilis* there was no evidence for *vnfX* downstream from *vnfN*. There was a small open reading frame following *vnfN* that showed no similarity to *nifX*, *vnfX*, or any sequence in the GenBank database. An open reading frame found in the opposite orientation just downstream from *vnfN* showed about 50% amino acid identity (80% similarity) to a putative protein of *Synechocystis* sp. of unknown function (GenBank accession number S110658) (data not shown). Thus, there is no evidence for additional genes that are part of the *vnfEN* cluster in *A. variabilis*. Northern analysis of RNA isolated from cells grown with or without Mo or V and hybridized to a *vnfEN*-specific probe indicated that the *vnfEN* genes, like the *vnfDGK* genes (36), were transcribed in the absence of Mo, whether or not V was present (data not shown).

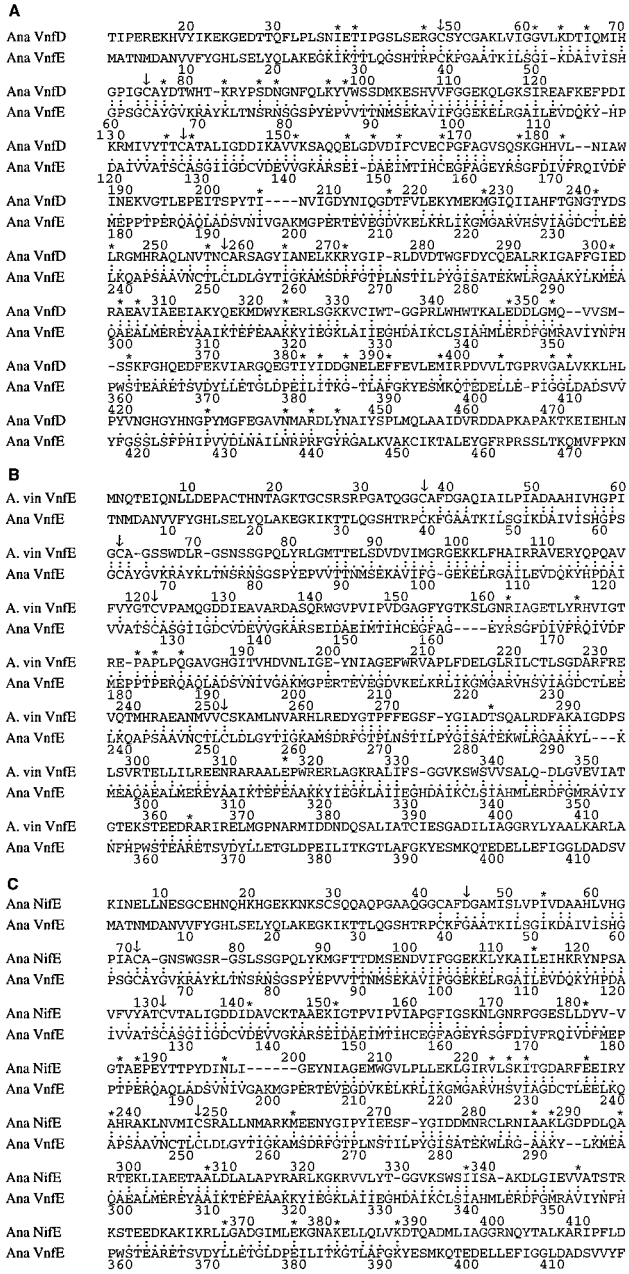
Comparisons of the deduced amino acid sequences of *vnfE* and *vnfN* with the *vnfE* and *vnfN* genes of *A. vinelandii* and with the *nifE* and *nifN* genes of *Anabaena* sp. PCC 7120 revealed only moderate similarity (Table 2). (The *nifEN* genes of *Anabaena* sp. PCC 7120 were used for comparison because the *nifEN1* genes of *A. variabilis* have not been completely sequenced. However, a part of *nifEN1* that has been sequenced has the same amino acid sequence as that region in *Anabaena* sp. PCC 7120 [39], and over 3 kb in the *nifBSU1* region of *A. variabilis* showed greater than 95% nucleotide identity with the homologous region from *Anabaena* sp. PCC 7120 [25].) The greatest similarities were not between the *vnfEN* genes of *A. variabilis* and *A. vinelandii* but between the *vnfE* and *vnfD* genes of *A. variabilis* and between the *vnfN* and *vnfK* genes of this organism. There was also good similarity between *vnfE* and *nifE* of *Anabaena* sp. PCC 7120; however, there was almost no similarity between *vnfN* and *nifN*.

Comparisons between the two *vnfE* genes and *nifE* revealed that 38 residues were conserved in all three sequences, including four cysteines that are indicated by arrows above the amino acids in Fig. 2. Since conservation of residues in all four sequences is presumably dictated by NifE/VnfE function, these residues are unlikely to provide information concerning the origin of the genes. Other amino acid similarities that are unique to each pair, and thus are probably not important for function, can provide evidence for the origin of these genes. Such amino acids are indicated by asterisks above the amino acids in Fig. 2. Of the 101 identical amino acids in *vnfE* and *vnfD* of *A. variabilis*, 48 were shared only between these two sequences (Fig. 2A). Particularly striking was a conserved additional residue, tyrosine, at position 65 of *vnfE* that was also present in *vnfD* (Fig. 2A). This residue was missing in the *vnfE* gene (Fig. 2B) of *A. vinelandii* and in *nifE* (Fig. 2C). The conservation of an apparently unnecessary residue in two sequences suggests a common ancestry; the probability that these two sequences would have acquired the tyrosine insertion independently is remote. Twenty-four amino acids were shared only by *vnfE* and *nifE* (Fig. 2C), and eight were shared only by *vnfE* from *A. variabilis* and *vnfE* from *A. vinelandii* (Fig. 2B). It appeared that the *vnfE* gene of *A. variabilis* was much more like the *vnfD* gene just upstream than like the *vnfE* gene of *A. vinelandii*.

The *vnfN* gene of *A. variabilis* showed greater similarity to *vnfK* (Fig. 2D) than to the *vnfN* gene of *A. vinelandii* (Fig. 2E); however, unlike *vnfE* and *nifE*, there was very little similarity between *vnfN* and *nifN* (comparison not shown). Thirty-two amino acids were common to the two *vnfN* genes and *vnfK*; however, *nifN* shared only one of these. There were 64 residues (indicated by asterisks in Fig. 2) shared only by *vnfN* and *vnfK* (Fig. 2D), while 40 were shared only by the *vnfN* genes of *A. variabilis* and *A. vinelandii* (Fig. 2E). A single cysteine was common to the two *vnfN* genes and *vnfK*, and this cysteine was not found in *nifN*. Just as the *vnfE* gene was most like the *vnfD* gene, the *vnfN* gene was most like the *vnfK* gene upstream.

Construction of a *vnfN* mutant. Mutant strain TT220 was created by replacement of the wild-type *vnfN* gene in the chromosome of *A. variabilis* by *vnfN* containing an insertion of the Nm^r gene (*npt*) from Tn*5* in the *Xba*I site (Fig. 1A). The replacement of the wild-type allele by the mutation was confirmed by Southern analysis of DNA isolated from the parent and mutant strains, using a probe that spanned the region containing the insertion. Addition of the 1.1-kb Nm^r gene increased the size of the hybridizing *Cla*I fragment from about 9.5 in the parent to 10.6 kb in the mutant strain TT220 (Fig. 1C, lanes 1 and 3). The probe spanned two *Eco*RV fragments of about 1.7 and 2.8 kb in the parent strain; addition of the 1.1-kb Nm^r gene resulted in hybridization of the probe to two bands of about equal size in the mutant (Fig. 1C, lanes 4 and 6).

Complementation was accomplished by integration of a wild-type copy of *vnfN* upstream of the mutation by single recombination between a nonreplicating plasmid, carrying the wild-type allele on an *Eco*RI fragment, and the chromosome of the mutant strain TT220, producing strain TT220-237 (Fig. 1B). The correct insertion of the wild-type copy in the chromosome of TT220-237 was confirmed by Southern analysis of DNA isolated from that strain. The additional copy of the wild-type region upstream of the mutated copy resulted in the introduction of a new *Cla*I site, derived from the vector, that was about 8.1 kb from the *Cla*I site located in *vnfK*, whereas the *Cla*I site in the chromosome of the parent strain, FD, was about 9.5 kb from the *Cla*I site in *vnfK* (Fig. 1B and C, lanes 1 and 2). The mutated copy of *vnfN*, containing the Nm^r gene, was unaltered in TT220-237, giving rise to a *Cla*I fragment of about 10.6 kb that hybridized to the probe (Fig. 1C, lane 2). The wild-type hybridizing *Eco*RV fragment of 1.7 kb was restored in TT220-237, which also retained the doublet at about 2.8 kb seen in the mutant strain TT220 (Fig. 1C, lanes 5 and 6).



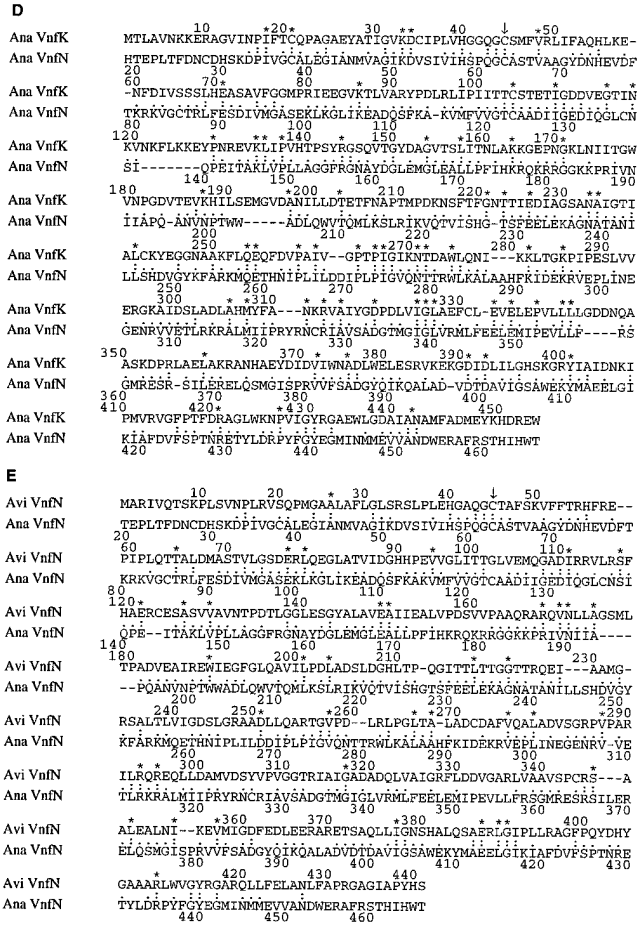


FIG. 2. Comparison of deduced amino acid sequences of VnfD from *A. variabilis* (Ana) and VnfE from the same strain (A), VnfE from *A. vinelandii* (A. vin) and VnfE from *A. variabilis* (B), NifE from *Anabaena* sp. strain PCC 7120 (Ana) and VnfE from *A. variabilis* (C), VnfK from *A. variabilis* and VnfN from the same strain (D), and VnfK from *A. vinelandii* and VnfN from *A. variabilis* (E). Double vertical dots indicate identical amino acids, and single dots indicate functionally similar amino acids; asterisks indicate amino acids shared uniquely by the pairs indicated, and the arrows indicate cysteines shared by all VnfE pairs or both VnfN pairs.

Growth and acetylene reduction. The parent strain, FD, and the strain containing both wild-type and mutant *vnfN* alleles, TT220-237, grew well in the presence of Mo or V (Fig. 3A and B) but grew poorly in the absence of both metals (Fig. 3C). The *vnfN* mutant, TT220, and the previously characterized *vnfDG* mutant, MB2 (36), grew poorly in the absence of Mo, whether or not V was present (Fig. 3B and C). These results indicate that the *vnfN* mutant, like the *vnfDG* mutant, could not grow diazotrophically using the V-nitrogenase system.

Strain FD, grown with V, reduced acetylene to ethane, a characteristic of V-nitrogenases (5, 12, 13) (Table 3). Mutant strains TT220 and MB2, grown with V, reduced acetylene poorly, without detectable ethane, as did all four strains grown in the absence of Mo and V (Table 3). Complementation of the *vnfN* mutation in strain TT220-237 resulted in restoration of acetylene reduction in the presence of V to wild-type levels, with production of ethane (Table 3). These results confirmed that the mutation in the *vnfN* gene abolished V-nitrogenase function.

Comparison of acetylene reduction by the wild-type strain grown with Mo or with V indicated that the V-nitrogenase system reduced acetylene poorly compared with the Mo-nitrogenase (Table 3). The low specific activity of the V-nitrogenase compared with the Mo-nitrogenase was not accompanied by very slow growth for cells using the V-nitrogenase versus the Mo-nitrogenase (Fig. 3A and B); thus, the V-nitrogenase of *A. variabilis* apparently reduced N_2 better than it reduced acetylene. Surprisingly, acetylene reduction in wild-type cells grown in the absence of Mo and V was relatively high (Table 3), despite the very poor growth of cells in this medium (Fig. 3C). This medium had been both chemically and biologically scrubbed of Mo (see Materials and Methods). Addition of 10^{-9} M Mo to this medium restored normal Mo-dependent growth and acetylene reduction of strain FD, and addition of 10^{-10} M Mo significantly increased acetylene reduction by this

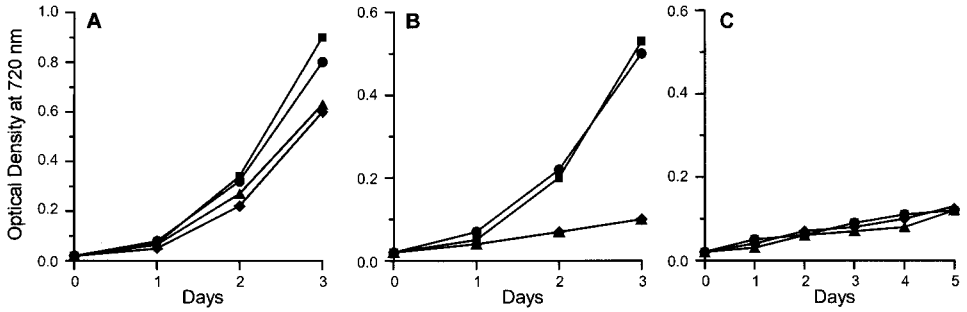


FIG. 3. Growth of parent, *vnf* mutant strains and the complemented strain of *A. variabilis* under nitrogen-fixing conditions with Mo (1.0 μM) (A), V (1.0 μM) (B), or neither metal (C) in the medium. ■, FD; ●, TT220-237; ▲, TT220; ◆, MB2.

strain; thus, the scrubbed medium contained less than 10^{-10} M Mo (data not shown).

DISCUSSION

Two genes downstream from *vnfDGK* encode proteins that are essential for V-nitrogenase function. Although not highly similar to other *nifEN* or *vnfEN* genes, there is sufficient sequence similarity to conclude that these are the *vnfEN* genes of *A. variabilis*. Four of the five cysteines conserved in *vnfE* and *nifE* in *A. vinelandii* (43) as well as in *nifE* of *Anabaena* sp. strain PCC 7120 were also conserved in the *vnfE* gene of *A. variabilis* (Fig. 2B and C). Similarly, the single cysteine that is conserved between *vnfN* and *nifN* genes in *A. vinelandii* (43) was also conserved in the *vnfN* gene of *A. variabilis* (Fig. 2E). That cysteine was not conserved between the *nifN* gene of *Anabaena* sp. PCC 7120 and the *vnfN* gene of *A. variabilis* (data not shown).

Amino acid comparison data provide evidence that the *vnfEN* genes of *A. variabilis* arose by duplication of the *vnfD* and *vnfK* genes of this strain. Because of the high degree of similarity of *vnfDGK* between *A. variabilis* and *A. vinelandii* (36), lateral transfer of the *vnfDGK* genes between *Azotobacter* and *Anabaena* species is a possibility. However, if the entire *vnfDGKEN* cluster was transferred, there should be similar values for sequence identity for all genes in the cluster. The discrepancy in similarity values for only the *vnfEN* genes and the similarity of *vnfE* to *vnfD* as well as *vnfN* to *vnfK* suggest that the *vnfEN* genes of *A. variabilis* did not come with the *vnfDGK* genes but rather arose by duplication of *vnfD* and *vnfK*. The rather low similarity values for *vnfE* compared with *vnfD* and *vnfN* compared with *vnfK* in *A. variabilis* further suggest that this duplication is ancient. The relatively high degree of similarity between the *vnfDGK* clusters of *A. vinelandii* and *A. variabilis* (65 to 86% amino acid similarity) (36) may simply reflect more stringent requirements for conservation of amino acids in the V-nitrogenase proteins than in the VnfEN proteins. The lack of *vnfX* downstream of *vnfEN* and of *vnfH* upstream of *vnfDGK* in *A. variabilis* also supports the idea that the *vnf* cluster did not move as a unit late in its evolution. The *vnfE* and *vnfN* genes of *A. vinelandii* are most like the *nifE* and *nifN* genes of the same strain (66 and 52% amino acid identity, respectively); *vnfE* has only 27% amino acid identity with *vnfD*, and *vnfN* has 28% amino acid identity with *vnfK* (43). Therefore, the *vnfEN* genes of *A. vinelandii* are most closely related to *nifEN* (43) and apparently did not arise by duplication of *vnfDK*. Although these various data suggest that the *vnf* gene cluster in *Anabaena* species separated from the cluster in *Azotobacter* species long ago, probably before the *vnfEN* genes evolved in either strain, more definitive answers await a quantitative phylogenetic analysis of these genes.

There are also functional and regulatory differences between the *vnfEN* genes of *A. variabilis* and *A. vinelandii*. While the function of the VnfEN proteins can be fulfilled by the NifEN proteins in *A. vinelandii* (43), the VnfEN proteins were essential for V-dependent nitrogen fixation in *A. variabilis*. The *vnf* clusters of the two organisms also appear to be regulated differently. The *vnf* genes of *A. vinelandii* are regulated by the products of *vnfA* and *rpoN* (14, 19, 23, 41). We can find no evidence for a *vnfA*-like gene in *A. variabilis* by Southern hybridization or by PCR amplification using primers for highly conserved regions of *vnfA* and *nifA*. Although PCR products of appropriate sizes were amplified, sequence analysis showed the sequences to be unrelated to *nifA* or *vnfA* (37). The dependence of V-nitrogenase of *A. variabilis* on a functional *nifB1* gene (25) suggests that the *vnf* genes, like the *nif1* genes, are regulated by factors that control gene expression after heterocyst differentiation, a mechanism that is likely to be very different from control in other diazotrophic bacteria.

Acetylene reduction is a simple, sensitive assay for nitrogenase activity; however, it may not accurately reflect nitrogen fixation. Mylona et al. (26) found that mutants in two genes of the *anf* system of *A. vinelandii*, which encodes the non-Mo, non-V nitrogenase, have high acetylene reduction activities but

TABLE 3. Acetylene reduction by wild-type and *vnf* mutant strains

Strain (genotype)	Ethylene (nmol/OD_{720}/h)[a] production by strains grown in medium of the indicated composition		
	+Mo	+V	−Mo −V
FD (wild type)	12.9 ± 2.3	3.2 ± 0.8 (0.08 ± 0.015)	1.1 ± 0.4
MB2 (*vnfDG*::*npt*)	10.5 ± 0.8	1.1 ± 0.2	1.2 ± 0.3
TT220 (*vnfN*::*npt*)	11.2 ± 1.4	0.7 ± 0.2	0.8 ± 0.1
TT220-237 (*vnfN*::*npt*/*vnfN*$^+$)	11.6 ± 1.2	3.2 ± 0.7 (0.08 ± 0.016)	0.7 ± 0.3

[a] Mean and standard deviation for three to four experiments with duplicate samples. Values in parentheses indicate ethane (nanomoles per OD_{720} per hour) production in only two samples.

fail to grow. Similarly, mutants in the *anfG* or *vnfG* gene of *A. vinelandii* fail to grow but retain various amounts of acetylene reduction activity (42). In *A. variabilis*, both wild-type and *vnf* mutant strains grew very poorly in the absence of Mo and V; however, acetylene reduction values were about one-third of the values for V-grown cells. Thus, nitrogenase synthesized under these conditions reduced acetylene, but the enzyme could not reduce sufficient N_2 to support good diazotrophic growth. The starvation for fixed nitrogen in these cultures results in degradation of pigments and an increased frequency in heterocysts from a normal value of about 5% to 15 to 20% in cells starved for both Mo and V (36). While a distinct non-Mo, non-V nitrogenase could account for the acetylene reduction observed in cells grown without metal, that seems unlikely given the very poor growth of cells in metal-deficient medium.

In this and in a previous study (36), *A. variabilis* grew nearly as well with the V-nitrogenase as with the Mo-nitrogenase: the generation time, during the period of most rapid growth, was about 25% longer for cells using the V-nitrogenase than for cells using the Mo-nitrogenase. However, acetylene reduction values suggested that the V-nitrogenase was only about a quarter as efficient as the Mo-nitrogenase (Table 2). Thus, the V-nitrogenase reduced acetylene poorly compared with the Mo-nitrogenase but, as judged from growth rates, reduced dinitrogen well.

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